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Linkage and Expression of Foreign DNA in Cultured Animal Cells

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The ability to introduce defined genes into cultured cells radically alters the prospects for understanding differentiation and growth control in higher organisms. Stable transformation of animal cells may be effected by the delivery of DNA as a calcium phosphate coprecipitate (Graham and van der Eb 1973). Since the uptake and expression of DNA is a relatively rare event, transformants are usually isolated by selection for cells that have acquired a new phenotype. For this purpose the thymidine kinase *(tk)*[1] gene of herpes simplex virus type 1 (HSV-1) is often used as the selectable vector with tk^- recipient cells (Wigler et al. 1977). The tk^+ transformants are then selected in HAT medium. Nonselectable genes may be stably introduced into cells either by direct ligation to the selectable vector (Mantei et al. 1979) or by unlinked cotransformation (Wigler et al. 1979b) (see below).

In this paper we characterize the DNA transfer system with respect to the physical fate and function of exogenously acquired DNA in transformed hosts and discuss applications of our observations to somatic cell genetics.

Linkage of Exogenous DNA in Transformants

Efficient transfer of DNA by the calcium phosphate method requires the use of "carrier" DNA that presumably facilitates the formation of a calcium phosphate-DNA coprecipitate suitable for cellular uptake (Graham and van der Eb 1973). Cells that stably incorporate and express a selectable DNA marker are also likely to incorporate stably other DNA sequences present among the carrier DNA (Wigler et al. 1979b). These exogenously acquired sequences are linked within the host cell to the selectable marker, and some of the evidence for this statement is discussed below.

Loss of cotransformed sequences upon reversion of the transformed phenotype. Revertants of tk^+ transformants can be selected by cloning cells in medium containing bromodeoxyuridine (BrdU). tk^+ transformants fall into two classes: those that clone in BrdU with high efficiency (greater than 5% of the cloning efficiency in neutral medium) and those that clone in BrdU but with lower efficiency (between 1% and 0.01%). In the latter case, blot analysis indicates that the revertant colonies that arise have deleted the transforming *tk* sequences (Perucho et al. 1980a; see Fig. 1). The fate of incorporated carrier sequences in revertants can also be examined by blot hybridization when a defined sequence has been included along with the carrier sequences during the initial transformation. The fate of the defined sequences can then be followed and taken as representative of other carrier sequences.

One example of such a study is illustrated in Figure 1. A transformed cell line, ϕX-4, was obtained from mouse Ltk^- recipients that contained a single copy of the HSV-1 *tk* gene and approximately 25 copies of bacteriophage ϕX174 Rf DNA (Wigler et al. 1979b). Twenty BrdU-selected revertants of ϕX-4 were cloned along with several tk^+ subclones grown in HAT medium. The tk^+ subclones still contain *tk* gene in the same immediate sequence environment as the parental, whereas the revertants have lost all traces of *tk* gene sequences (Fig. 1b). Of the 20 revertants, 8 have also lost all traces of ϕX174 sequences, whereas the remaining 12 revertants retain some, but not all, of the ϕX174 sequences that were formerly present. tk^+ subclones, on the other hand, retain the ϕX174 blot pattern of the parental line ϕX-4. This sort of experiment has yielded similar results using cells of different origins, with different selectable markers, and with other cotransformed hybridization markers (Perucho et al. 1980a). In each case, at least 50% of all revertants have lost all cotransformed sequences, whereas all subclones selected for retention of the transformed phenotype retain virtually all cotransformed sequences.

Physical linkage of foreign sequences. The simplest interpretation of this result is that all exogenously acquired DNA in transformed host cells is physically linked and that reversion arising by deletion of the selectable element leads to the deletion of some or all of the other foreign sequences. Direct confirmation of this prediction has been given previously (Perucho et al. 1980a). In brief, bacterial plasmid derivatives of pBR322 were cotransformed into mouse cells using the *tk* gene as the selectable marker and either salmon or hamster genomic DNA as carrier. These pBR322 sequences were then rescued from their animal cell hosts by restriction cleavage, cyclization with T4 DNA ligase, and transformation into *Escherichia coli.* The resultant

[1] Nomenclature adopted in this paper is to designate genotype in lower-case italic letters (e.g., *tk*), phenotype in lower-case roman letters (e.g., tk), and gene product or protein in upper-case roman letters (e.g., TK).

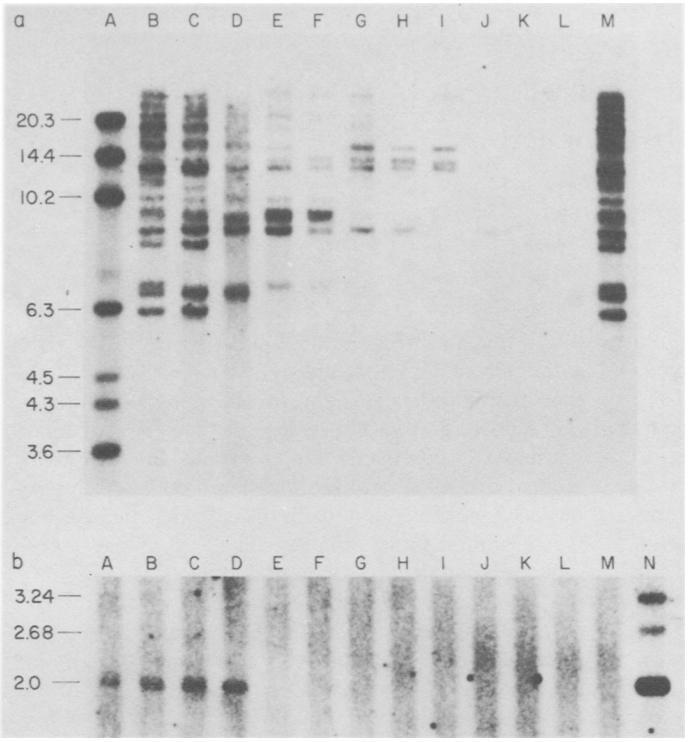

Figure 1. Annealing pattern of cotransformed ϕX174 sequences in tk$^-$ revertants of ϕX-4. (*a*) tk$^-$ revertants from the line ϕX-4 were isolated by subcloning in medium containing BrdU, and high-molecular-weight DNA was prepared from each of these subclones. 10 μg of each DNA was cleaved with *Hin*dIII, electrophoresed in a 0.8% agarose slab gel, and analyzed by blot hybridization using ^{32}P-labeled, nick-translated ϕX174 DNA (4×10^8 cpm per μg) as probe. (Lane *A*) Mixture of *Eco*RI and *Bam*HI digests of ^{32}P-labeled Ad2 DNA; the molecular weights (in kb) of these fragments are at left; (lanes *B* and *M*) ϕX-4 parental line; (lanes *C–L*) ten different subclones resistant to BrdU. (*b*) 10 μg of DNA from different subclones in HAT (lanes *A–D*) or BrdU (lanes *E–L*) from the line ϕX-4 were digested with *Pvu*II and electrophoresed through 0.8% agarose gels. Blot analysis was performed using as probe the nick-translated, purified 3.5-kb *Bam*HI fragment of ptk2 containing the HSV-1 *tk* gene (1×10^8 cpm per μg) (Hanahan et al. 1980). (Lane *M*) 10 μg of Ltk$^-$ DNA digested with *Pvu*II; (lane *N*) 60 pg of *Pvu*II-cleaved ptk2; lines at left indicate the molecular weights of these ptk2 fragments.

"rescuants" contained not only pBR322 sequences, but also those sequences that flanked them in their previous host. In each case examined, these flanking sequences were analyzed by blot and solution hybridization and shown to derive from the species of the carrier rather than from mouse. We conclude from this study that animal cells that take up DNA fragments rapidly ligate them into a possibly very large concatameric structure, which we have previously named a pekelasome (Perucho et al. 1980a). The work described in the previous section implies that cells usually contain only a single pekelasome (since most times only a single copy of the selectable gene is present). We further infer from this that all the exogenously acquired DNA that is stably retained in a transformant is so retained by virtue of its linkage to the selectable element.

Expression of *tk* Genes Introduced into Cells by Cotransformation

As we have amply indicated, cells that take up a selected marker also incorporate other nonselected sequences. For cotransformation to be of use in studying the expression of genes, we need to know whether such genes are usually or only occasionally expressed. There is conflict in the literature; for example, whether the rabbit β-globin gene expresses when cotransformed into Ltk$^-$ cells (Mantei et al. 1979; Wold et al. 1979). In the following experiments we have utilized the cloned HSV-1 *tk* gene and the cloned chicken *tk* gene as model systems and have introduced these genes into the double mutant Ltk$^-$aprt$^-$ recipient without selection for expression by using either the dihydrofolate reductase (*dhfr*) or adenine phosphoribosyl transferase (*aprt*) genes as the selectable agent. We have then studied the expression of the *tk* genes in these cotransformants by measuring cloning efficiencies in HAT medium.

Expression of HSV-1* tk *genes in cotransformed cells. Ltk$^-$aprt$^-$ cells were transformed for the aprt$^+$ phenotype with chicken genomic DNA and cotransformed with the cloned HSV-1 *tk* gene. aprt$^+$ transformants were selected in AA medium, containing azaserine and adenine (Wigler et al. 1979a), and individual clones were grown into mass culture. After growth in AA medium for about 25 generations, the cloning efficiencies of the aprt$^+$ transformants were measured in various media. Only those that contained and expressed the *tk* gene would be able to survive in HAT medium. Of the 27 aprt$^+$ clones analyzed, 9 grew in the selective medium for TK (Table 1). When analyzed by filter hybridization using the *Eco*RI fragment of the HSV-1 *tk* gene as probe, 10 clones of the original 27 contained sequences homologous to *tk*, and the other 17 were negative (Fig. 2). Eight of the positive clones contained an homologous hybridizing band comigrating with the *Pvu*II fragment of ptk2 that comprises the entire HSV-1 *tk* gene (Colbere-Garapin et al. 1979). These same 8 clones were the ones that grew in HAT medium with high plating efficiency (Table 1). Clones 10 and 24 failed to grow in HAT medium and contained HSV-1 *tk* gene sequences but not the 2.2-kb *Pvu*II fragment. They were further analyzed by digestion with *Sma*I and *Eco*RI, which give rise to a 1.23-kb fragment of the HSV-1 *tk*

Table 1. Cotransformation and Expression of the HSV-1 *tk* Gene in Ltk$^-$aprt$^-$ Cells

Clone	Growth in HAT[a]	Relative cloning efficiencies[b]			HSV-1 *tk* gene content[c]
		DMEM	AA	HAT	
1	−	—	—	—	−
2	+	100	50	100	+
3	−	—	—	—	−
4	−	—	—	—	−
5	+	100	55	95	+
6	−	—	—	—	−
7	−	—	—	—	−
8	+	100	50	98	+
9	−	—	—	—	−
10	−	—	—	—	(±)
11	−	—	—	—	−
12	−	—	—	—	−
13	−	—	—	—	−
14	+	100	44	97	+
15	+	100	68	1.5	(±)
16	+	100	95	100	+
17	−	—	—	—	−
18	−	—	—	—	−
19	+	100	25	96	+
20	−	—	—	—	−
21	−	—	—	—	−
22	−	—	—	—	−
23	−	—	—	—	−
24	−	—	—	—	(±)
25	+	100	60	90	+
26	−	—	—	—	−
27	+	100	95	110	+

[a] 10^4–10^5 cells from 27 independent aprt$^+$ transformants derived from cells transformed to aprt$^+$ with chicken DNA in the presence of *Sal*I linearized ptk2, the cloned HSV-1 *tk* gene (Hanahan et al. 1980), were plated in HAT medium. "+" indicates that at least one HAT-resistant colony emerged. The cloning efficiency of HAT "+" clones was then quantitated.

[b] 10^2–10^3 cells were plated in neutral medium (DMEM) or aprt$^+$ selective medium containing azazerine and adenine (AA) or tk$^+$ selective medium (HAT). Cloning efficiencies are given relative to that in neutral medium.

[c] The presence of HSV-1 *tk* gene sequences in transformants was examined by blot hybridization. "+" indicates at least one copy per cell of a *complete* HSV-1 *tk* gene. "±" indicates either less than a full-length gene was present (clones 24 and 10) or that a full-length gene was present but at less than a single copy per cell (clone 15). See text for details.

gene (Colbere-Garapin et al. 1979), which is close to the minimum needed for TK expression. Blot analysis of these clones is shown in Figure 3. Although all three HAT-positive clones (2, 14, and 16) contained the 1.23-kb *Eco*RI/*Sma*I fragment of the HSV-1 *tk* gene, clones 10 and 24 did not contain this fragment. Thus, 8 of 8 cells with a complete HSV-1 *tk* genome grow in HAT medium with cloning efficiencies similar to that in neutral medium. From this we conclude that most cells in populations containing the HSV-1 *tk* gene express that gene even without a prior history of selection for its expression.

One exceptional clone, number 15, displayed a low plating efficiency in the selective medium for TK yet, by blot analysis, did not appear to contain the complete HSV-1 *tk* gene. Several explanations for this were possible: The cell population was slightly contaminated with another tk$^+$ cell line; a barely functional *tk* fragment was expressing at low levels; or only a small percentage of cells retained a complete HSV-1 *tk* gene after growth in the absence of selective pressure for it. To resolve these questions, cells from clone 15 were grown in AA or HAT medium for about 30 generations and analyzed for HSV-1 *tk* gene sequence content by blot hybridization. Like the cells grown in AA medium, the cells that were selected by growth in HAT medium contained an incomplete HSV-1 *tk* gene fragment (a 1.4-kb *Pvu*II fragment and a 4-kb *Eco*RI/*Sma*I fragment, lanes E, F, G, and H of Fig. 4), demonstrating a common parental transformant for the two cell populations. But unlike the cells grown in the original APRT selective medium, cells selected for the *tk* gene also contained complete HSV-1 *tk*-coding sequences, as indicated by the presence of both the 2.2-kb *Pvu*II fragment and the 1.23-kb *Eco*RI/*Sma*I fragments (lanes F and H, Fig. 4). These results indicate that the presence of the HSV-1 *tk* gene was unstable in this aprt$^+$ transformant, possibly because it was not genetically linked to the *aprt* gene. The behavior of clone 15 also indicates that the recipient Ltk$^-$aprt$^-$ cells can at least occasionally incorporate more than one pekelasome.

The results described strongly suggest that the HSV-1 *tk* gene, introduced by cotransformation into Ltk$^-$aprt$^-$ cells and grown in the absence of selective pressure for it, is constitutively expressed (notice the high plating efficiency in HAT medium of cells from clones 2, 5, 8, 14, 16, 19, 25, and 27 grown in AA medium), and therefore its expression is not limited by any especially favorable environment in the pekelasome. A further conclusion from these studies is that the cloned 3.5-kb *Bam*HI frag-

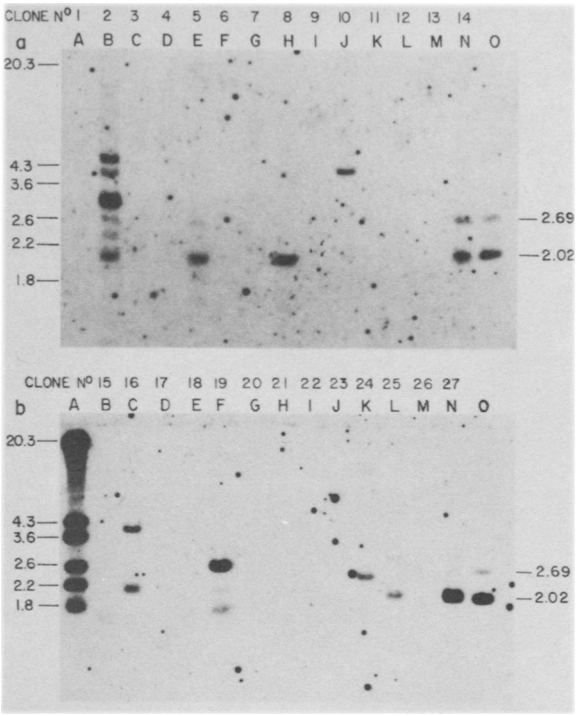

Figure 2. Blotting pattern of the HSV-1 *tk* gene in cotransformed Ltk$^-$aprt$^-$ cells. Ltk$^-$aprt$^-$ cells were transformed with chicken genomic DNA in the presence of *Sal*I-cleaved ptk2. aprt$^+$ transformants were selected in AA medium. Cellular DNA from each of 27 chicken aprt$^+$ Ltk$^-$aprt$^-$ clones grown in AA medium were digested with *Pvu*II, electrophoresed through 1% agarose gels, and analyzed by blot hybridization using the 2.4-kb *Eco*RI fragment of ptk2, ^{32}P-labeled by nick translation (2×10^8 cpm/μg), as probe. (Lanes *A–N* in *a* and lanes *B–N* in *b*) DNA from the 27 clones (10 μg each) digested with *Pvu*II; (lane *A* in *b*) ^{32}P-labeled Ad2 DNA cleaved with *Eco*RI as size markers; (lanes *O*) 50 pg of *Pvu*II-cleaved ptk2 with 5 μg of Ltk$^-$aprt$^-$ DNA as carrier. The lines at left indicate the sizes (in kb) of the Ad2 DNA *Eco*RI fragments, and the lines at right indicate the molecular weights of the *Pvu*II fragments of ptk2 hybridizing with the *Eco*RI fragment used as probe.

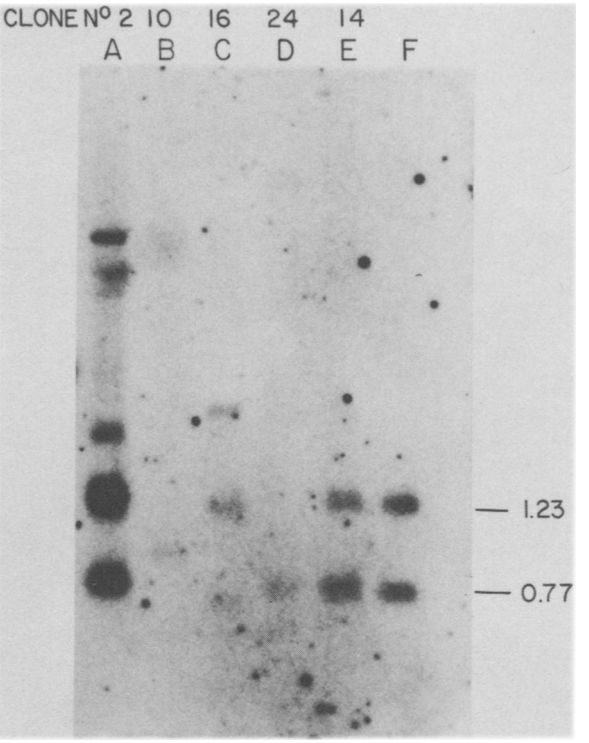

Figure 3. Presence of the *Sma*I/*Eco*RI fragment of the HSV-1 *tk* gene in Ltk$^-$aprt$^-$ chicken aprt$^+$ clones. Cellular DNA from different chicken aprt$^+$ clones grown in AA medium were digested with *Eco*RI and *Sma*I, electrophoresed through 1% agarose gels, and analyzed by blot hybridization as described in the legend to Fig. 2. (*A–E*) 10 μg of DNA from clones 2, 10, 18, 27, and 15 (Fig. 2), respectively; (*F*) 50 pg of *Sma*I/*Eco*RI-cleaved ptk2 with 5 μg Ltk$^-$aprt$^-$ DNA as carrier. At right is indicated the size in kb of the two *Eco*RI/*Sma*I fragments of ptk2 hybridizing with the 2.4-kb *Eco*RI fragment used as probe.

ment of HSV-1 encodes a complete *tk* gene that requires no special recombination with cellular sequences in order to express.

Expression of the chicken* tk *gene in cotransformed cells. The expression of the chicken *tk* gene introduced by cotransformation in Ltk$^-$aprt$^-$ cells was studied in a fashion similar to that used for the HSV-1 *tk* gene, but using another vector as selectable marker, the mutant *dhfr* gene of the hamster line A29 (Wigler et al. 1980), which is resistant to low levels of methotrexate (MTX). Ltk$^-$aprt$^-$ cells were transformed for the resistance to MTX using genomic DNA from the line A29 (20 μg per dish) and cotransformed with 1 μg per dish of circular pchtk2, a plasmid containing the *Eco*RI/*Hin*dIII 2.2-kb fragment coding for the chicken *tk* gene (Perucho et al. 1980b). Twenty-two clones resistant to 0.1 μg per ml of MTX were isolated, grown into mass culture for about 15 generations, and then plated into either HM medium, containing hypoxanthine and 40 μg/ml of MTX, or HTM medium, containing additional thymidine. Clones will grow in HTM if they either are expressing the chicken *tk* gene or have spontaneously amplifed the donor *dhfr* gene, rendering them resistant to high levels of MTX (Wigler et al. 1980). In the latter case, cells will also grow in HM medium. Clones that grew in either medium were analyzed more carefully for their cloning efficiencies in DMEM (neutral medium), HM, or HTM medium. The results are summarized in Table 2.

Clones 1, 3, 6, 13, 14, and 20 showed no growth in either HM or HTM medium. Clones 2, 15, 16, and 17 cloned with low efficiency in HM medium, presumably due to the emergence of a subpopulation of cells that had amplified the transferred *dhfr* gene, thereby becoming resistant to the higher MTX concentration. Although clones 2, 15, and 17 cloned no more efficiently in HTM than in HM medium, clone 16 showed a high efficiency of cloning in HTM medium. The other clones studied (4, 5, 7, 8, 9, 10, 11, 12, 18, 19, 21, 22) were able to grow in the selective medium for TK with plating efficiencies similar to the corresponding efficiencies in neutral medium but grew poorly or not at all in HM medium.

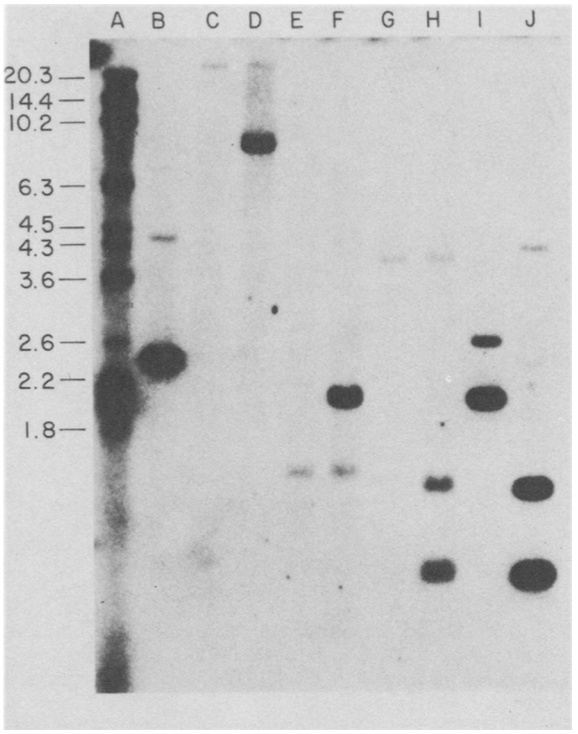

Figure 4. Blot hybridization analysis of HSV-1 *tk* sequences in clone 15 (Fig. 2) grown in AA or HAT medium. Cells from the aprt$^+$ clone 15 were grown in AA medium for 25 generations and transferred at that time to HAT medium. Cellular DNA was extracted from cells maintained for about 30 generations in either AA (*C, E,* and *G*) or HAT (*D, F,* and *H*) medium and digested with *Eco*RI (*C* and *D*), *Pvu*II (*E* and *F*), and *Eco*RI/*Sma*I (*G* and *H*), electrophoresed through 1% agarose gels, and analyzed by filter hybridization as described in the legend to Fig. 2. (*A*) Mixture of *Eco*RI and *Bam*HI digests of ^{32}P-labeled Ad2 DNA. The sizes in kb of the bands are indicated. (*B, I,* and *J*) *Eco*RI, *Pvu*II, and *Eco*RI/*Sma*I digests of ptk2, respectively (80 pg each), with 5 μg of Ltk$^-$aprt$^-$ DNA as carrier.

When analyzed by blot hybridization, using the chicken *tk* gene as probe (Fig. 5), clones 1, 2, 6, 13, 14, 15, 17, and 20, all HTM-growth-negative, did not show any detectable sequences strongly homologous to the chicken *tk* gene. On the other hand, clones 4, 5, 7, 9, 10, 11, 12, 18, 19, 21, and 22, which grew well in the selective medium for TK, contained discrete fragments homologous to the chicken *tk* gene (Figs. 5 and 6). Clone 3, which did not grow in HTM medium but contained chicken *tk* sequences, and clones 8 and 16, which did not grow in HTM medium with high plating efficiency and showed weakly hybridizing bands homologous to the chicken *tk* gene, were further analyzed by blot hybridization after double digestion with *Kpn*I/*Pvu*II, which generates a 1.26-kb fragment coding for an essential region of the chicken *tk* gene (Fig. 6). Clones 7 and 21, included in the blot as positive controls, showed a strong hybridizing band comigrating with the *Kpn*I/*Pvu*II fragment of pchtk2, whereas clones 8 and 16 showed a faint, but distinguishable, band of the same mobility. Clone 3 did not contain this fragment.

These results, like those presented before for the HSV-1 *tk* gene, indicate that the cells that incorporate a full-length chicken *tk* gene by cotransformation are able to express the *tk* gene even in the absence of any history of selection for its expression. The low cloning efficiency in HTM of clones 8 and 16 perhaps reflects a situation similar to that of clone 15 of the HSV-1 *tk* gene experiment.

Linkage of Cotransformed Phenotypes

Cellular genes can be transferred to appropriate recipient cells when total cellular DNA is used as donor. This has been demonstrated unequivocally for several selectable cellular markers such as TK and APRT (Wigler et al. 1978, 1979a). In these cases, discriminating selection schema were available, the recipient cells used had low or undetectable spontaneous mutation to the selected phenotype, and biochemical characterization of recipients allowed an unambiguous proof of gene transfer. For most phenotypes, these ideal conditions are usually not met. We may, however, exploit our previous observations to facilitate the detection of cellular gene transfer either when there is a high background of spontaneous mutation or when the phenotypic detection of transformants is otherwise difficult. The idea involves the use of a cell that is also tk$^-$ as recipient for the desired phenotype. The transformation with cellular donor DNA is then done in the presence of sufficient cloned *tk* gene to ensure at least a 50% frequency of cotransformation for both phenotypic markers. Cells transformed for one marker can then be screened for transformation for the other marker. The probability of finding a transformant for one marker that has spontaneously mutated for the other marker is far less than the frequency of spontaneous mutation itself. In addition, preselecting for the tk$^+$ transformants narrows the population of cells that needs to be screened for the other marker and therefore facilitates its detection. Finally, demonstration of genetic linkage between the two acquired markers provides definitive evidence of gene transfer. We illustrate the application of this approach below.

It has been demonstrated recently that DNA from certain growth-transformed[2] cell lines can be used as donor to confer growth transformation on otherwise growth-controlled recipient cells (Shih et al. 1979; Cooper et al. 1980). These donors contain putative dominant-acting "tumor" genes. The transformed clone is detected by its ability to give rise to a morphologically altered focus seen against a background of confluent normal cells. The main problem in the study of cellular "tumor" genes is the uncertainty of the origin of the foci of growth-transformed cells: Do foci arise from a transformation event dependent on exogenous DNA, or do they arise through some other mechanism? We have

[2] A confusion of nomenclature is unavoidable here. We use transformation to mean the process of introducing foreign DNA into cells and growth transformation to mean any of a variety of abnormal cellular growth phenotypes associated with tumorigenicity.

Table 2. Cotransformation and Expression of the Chicken *tk* Gene in Ltk$^-$aprt$^-$ Cells

Clone	Growth in[a]		Relative cloning efficiencies[b]			Chicken *tk* gene content[c]
	HM	HTM	DMEM	HM	HTM	
1	−	−	—	—	—	−
2	+	+	100	0.08	0.08	−
3	−	−	—	—	—	(±)
4	−	+	100	<0.01	65	+
5	−	+	100	<0.01	105	+
6	−	−	—	—	—	−
7	−	+	100	0.03	98	+
8	−	+	100	<0.01	16	(±)
9	−	+	100	0.02	85	+
10	−	+	nd	nd	nd	+
11	−	+	100	<0.01	95	+
12	−	+	100	<0.01	102	+
13	−	−	—	—	—	−
14	−	−	—	—	—	−
15	+	+	100	1.6	1.5	−
16	+	+	100	0.5	22	(±)
17	+	+	100	0.1	0.12	−
18	−	+	100	<0.01	96	+
19	−	+	100	0.02	78	+
20	−	−	−	—	—	−
21	−	+	nd	nd	nd	+
22	−	+	nd	nd	nd	+

[a] 10^5 cells from 22 independent clones resistant to low levels of MTX (0.1 μg/ml) derived from Ltk$^-$ cells transformed with A29 DNA in the presence of circular pchtk2, the cloned chicken *tk* gene (Perucho et al. 1980b), were plated into HM medium, containing hypoxanthine and MTX (40 μg/ml), or into HTM medium, containing hypoxanthine, thymidine, and MTX (40 μg/ml). Clones that gave rise to any colonies larger than 1 mm after 2 weeks' growth were scored positive.

[b] Clones that gave rise to colonies in the previous experiment were plated at varying concentrations in the media indicated and colonies larger than 1 mm counted 2 weeks later. Numbers are relative to cloning efficiency in DMEM, neutral medium. nd stands for not done.

[c] The presence of chicken *tk* gene sequences in transformants were examined by blot hybridization. "+" indicates at least one copy per cell of complete chicken *tk* gene sequences. "±" indicates either less than a full-length gene was present (clone 3) or a full-length gene was present but at less than one copy per cell (clones 8 and 16).

utilized double transformation with SV40 DNA and the chicken *tk* gene to work out a model system for the study of genes altering growth control.

Rat-2 is a tk$^-$ derivative of Rat-1, a morphologically normal rat fibroblast cell line (Botchan et al. 1976), which was isolated by W. Topp at Cold Spring Harbor. Rat-2, like its parent, exhibits "normal" growth features: low saturation density, high serum requirement, low

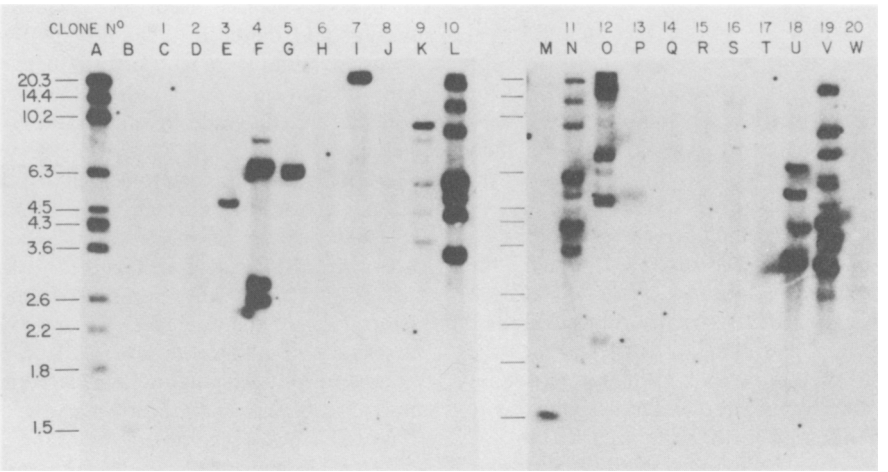

Figure 5. Blotting pattern of the chicken *tk* gene in cotransformed Ltk$^-$aprt$^-$ cells. Ltk$^-$aprt$^-$ cells were transformed for resistance to MTX using genomic DNA from the DHFR mutant line A29 and cotransformed with circular pchtk2. Twenty-two clones resistant to 0.1 μg per ml of MTX were isolated as described previously (Wigler et al. 1980). Cellular DNA from each of the clones grown in 0.1 μg per ml of MTX for about 20 generations was digested with *Hin*dIII, electrophoresed through 0.8% agarose gels, and analyzed by blot hybridization using the 1.1-kb *Sma*I/*Pvu*II fragment of the chicken *tk* gene, ^{32}P-labeled by nick translation (4×10^8 cpm/μg), as probe. (*A*) Mixture of *Eco*RI and *Bam*HI digests of ^{32}P-labeled Ad2 DNA; the sizes of the bands are indicated in kb. (*B* and *M*) 10 and 25 pg of the *Sma*I/*Pvu*II pchtk2 fragment, respectively, with 5 μg of Ltk$^-$aprt$^-$ DNA as carrier. (*C–L* and *N–W*) 20 different MTX-resistant Ltk$^-$aprt$^-$ derivatives (see Table 2).

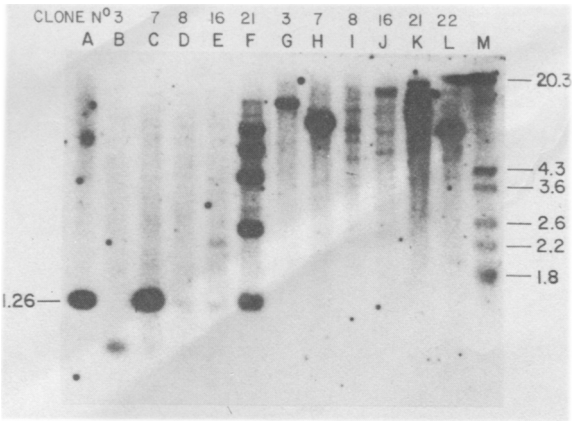

Figure 6. Presence of the 1.26-kb *Kpn*I/*Pvu*II fragment of the chicken *tk* gene in Ltk$^-$aprt$^-$ MTX-resistant clones. Cellular DNA from different Ltk$^-$aprt$^-$ MTX-resistant clones, grown in medium containing 0.1 μg/ml MTX, were digested with either *Kpn*I/*Pvu*II (*B–F*) or with *Bam*HI (*G–L*), electrophoresed through a 1% agarose gel, and analyzed by blot hybridization using the *Sma*I/*Pvu*II fragment of pchtk2 as probe (see Fig. 5). (*A*) 80 pg of pchtk2 cleaved with *Kpn*I/*Pvu*II, with 5 μg of Ltk$^-$aprt$^-$ DNA as carrier. The fragment sizes are indicated in kb; (*B–L*) 10 μg of DNA from the clones indicated on top; (*M*) *Eco*RI digest of ^{32}P-labeled Ad2 DNA. The sizes in kb of the fragments are indicated at right.

plating efficiency in suspension, and well-organized actin cables (W. Topp, unpubl.). At confluence, these cells form a flat, uniform monolayer with few, if any, spontaneous foci. These cells are further characterized by a high efficiency of DNA-mediated transformation with the calcium phosphate coprecipitation method: 10–30 foci of growth-transformed cells per ng of *Eco*RI-linearized SV40 DNA and 1–15 tk$^+$ transformants per ng of the cloned chicken *tk* gene can be obtained (Table 3). The efficiency of cotransformation is also high: When both SV40 DNA and chicken *tk* genes were included in the calcium phosphate precipitate, a high percentage of both SV40 and tk$^+$ transformants displayed the dual phenotype. From 17 foci picked at random, 14 contained cells able to grow in HAT medium, indicating that the SV40-transformed cells also incorporated the chicken *tk* gene. On the other hand, 5–10% of the tk$^+$ transformants selected first in HAT medium displayed a cellular morphology characteristic of SV40 transformants. The incorporation of SV40 sequences in these cells was demonstrated by immunofluorescence staining using a hamster antiserum specific for T antigen. All the isolated foci and nine out of ten morphologically altered tk$^+$ colonies were T-antigen-positive (Table 3).

We next examined the genetic linkage of the tk$^+$ and T-antigen-positive morphologically altered phenotypes in cotransformed cells. Rat-2 cells (Table 3) that were transformed with 20 ng per dish of *Eco*RI-cleaved SV40 DNA and 50 or 200 ng per dish of *Hin*dIII-digested pchtk5, a recombinant plasmid containing a 3-kb *Hin*dIII chicken DNA fragment containing the *tk* gene (Perucho et al. 1980b), were plated into neutral medium and into HAT medium. Both HAT-selected colonies, showing a transformed morphology and morphologically altered foci, containing cells capable of growing in HAT medium, were analyzed for T-antigen synthesis. Cell populations arising from the foci were practically homogeneously T-antigen-positive when grown in HAT medium, whereas the cells from foci plated in neutral medium were heterogeneous for T-antigen expression, indicating that the selection in HAT removed most of the surrounding nontransformed cells. Four colonies were subcloned from HAT-cultured populations and used for further study. Similarly, four colonies selected initially in HAT were again subcloned in HAT and studied further. These cloned populations were practically homogenously T-antigen-positive. All eight clones described were grown into mass culture, and tk$^-$ revert-

Table 3. Transformation and Cotransformation Efficiencies in Rat-2 Cells

DNA[a]			Morphologically altered foci			HAT-selected colonies		
SV40 *Eco*RI (ng/dish)	pchtk5 *Hin*dIII (ng/dish)	carrier (μg/dish)	foci/ng SV40	T-antigen-positive foci[b]	tk$^+$ foci[c]	colonies/ng pchtk5	morphologically altered colonies (%)[d]	T-antigen-positive colonies[e]
20	100	Ltk$^-$ (20)	1	5/5	7/7	5	5	—
—	100	Ltk$^-$ (20)	—	—	—	1.5	0	—
20	—	Ltk$^-$ (20)	10	10/10	—	—	—	—
20	200	Rat-2 (15)	1.5	5/5	2/5	1.0	5	4/5
—	200	Rat-2 (15)	—	—	—	1.0	0	—
20	50	Rat-2 (15)	9	5/5	5/5	12	10	5/5
—	50	Rat-2 (15)	—	—	—	12	0	—
20	—	Rat-2 (15)	30	3/3	0/5	—	—	—

[a] The indicated amounts of DNA were added directly to Rat-2 cells as a calcium phosphate coprecipitate as described previously (Wigler et al. 1980). 24 hr later, cells were trypsinized and split 1:3 into neutral medium for foci formation or into HAT medium for TK selection. Foci or tk$^+$ colonies were scored after 10–15 days. SV40 DNA was cleaved with *Eco*RI. pchtk5, a clone of the chicken *tk* gene, was linearized with *Hin*dIII.

[b] Morphologically altered foci were isolated by cloning cylinders and cells plated into both neutral medium and HAT medium. Cells were stained for SV40 T antigen using hamster antiserum, as described previously (Hanahan et al. 1980). The numbers are T-antigen-positive foci/foci examined.

[c] As above, except the numbers are foci containing HAT-resistant cells/foci examined.

[d] Percentage of tk$^+$ colonies that displayed an altered colony morphology characteristic of SV40-transformed cells.

[e] Individual morphologically altered tk$^+$ colonies were stained for T antigen. The numbers are T-antigen-positive colonies/colonies examined.

Table 4. Genetic Linkage of tk^+ and SV40 T-antigen-positive Phenotypes in Cotransformants

Transformant clone[a]	Medium	Cloning efficiency[b]	Morphology[c] T	I	N	T antigen[d]
	HAT	38	24	3	1	5/5
1a	DMEM	62	103	6	8	10/10
	BrdU	1.6	1	8	40	1/8
	HAT	32	6	0	0	5/5
2a	DMEM	49	9	2	1	14/14
	BrdU	3.0	1	3	9	2/19
	HAT	27	11	0	0	5/5
3a	DMEM	37	12	0	0	9/9
	BrdU	0.25	5	1	15	3/11
	HAT	24	7	0	0	5/5
4a	DMEM	40	6	1	0	9/9
	BrdU	0.37	6	5	15	8/21
	HAT	34	21	2	0	5/5
1b	DMEM	40	30	3	2	8/9
	BrdU	0.1	1	4	51	0/19
	HAT	40	24	2	0	5/5
2b	DMEM	50	33	1	3	5/5
	BrdU	2.4	1	22	61	2/11
	HAT	30	10	1	1	5/5
3b	DMEM	40	21	3	1	7/9
	BrdU	0.5	2	3	32	0/17
	HAT	46	29	0	0	5/5
4b	DMEM	50	26	0	1	6/6
	BrdU	0.5	10	1	150	1/15
Total	HAT		132	8	2	40/40 (100%)
	DMEM		240	16	16	68/71 (96%)
	BrdU		27	47	373	17/111 (15%)

[a] Four morphologically altered tk^+ foci (1a–4a) and four recloned HAT-selected tk^+ transformants (1b–4b) with altered morphology (see text) were grown into mass cultures in HAT medium, plated into HT medium (HAT medium lacking aminopterin), for 3 days; then 100 cells were plated into HAT medium and DMEM medium (neutral medium), and 2, 5, 10, and 50×10^3 cells into medium containing BrdU (30 μg/ml).

[b] Plating efficiency in the indicated medium: numbers of colonies after 14 days per 100 cells plated.

[c] Morphology of colonies was assessed after Giemsa staining: (T) SV40-type morphology; (N) normal (flat) morphology; (I) intermediate. Colonies too small to display characteristic morphologies were not scored.

[d] Colonies were picked at random and scored for T-antigen synthesis as described in the notes to Table 1. The correlations of morphology with T-antigen staining was as follows: 100% of colonies scored T were T-antigen-positive; 4.7% of colonies scored I were T-antigen-positive; and 2.1% of colonies scored N were T-antigen-positive.

ants were selected by growth in medium containing BrdU. Both the morphology of the colonies and T-antigen-synthesis pattern were compared with those of cells grown in parallel in neutral or HAT media (Table 4). All clones studied, four deriving from foci and four from tk^+ transformants, showed a similar behavior: Although cultures grown in HAT or neutral media maintained their SV40-transformed phenotype, with densely piled up, disorganized, T-antigen-positive cells, the selection in BrdU gave rise to a high percentage of normal colonies with flat, oriented, T-antigen-negative cells (Figs. 7 and 8; see note d, Table 4). With few exceptions, T-antigen-positive colonies were homogeneous for this character. Sometimes, colonies growing in neutral or BrdU-containing media presented a peculiar T-antigen pattern: Individual colonies were sectored for T-antigen-positive and -negative cells, possibly reflecting a stepwise loss of the transforming elements. No similar cases were observed when the cells were maintained in the presence of the selective pressure for the *tk* gene. When Rat-2 cells were transformed sequentially, first for SV40 transformation and then for *tk* transformation, no linkage was observed between phenotypes assayed as described above. These studies demonstrate the genetic linkage of the SV40 and chicken *tk* sequences when simultaneously cotransformed into Rat-2 cells. Recently, we have demonstrated the generality of these results for other viral transforming genes and have confidence in this approach to the study of growth-transforming genes of cellular origins.

CONCLUSIONS

Cultured cells can be efficiently transformed with DNA delivered as a calcium phosphate coprecipitate. Transformed cells, detected by the acquisition of a selectable phenotype, often incorporate other unlinked sequences present in the precipitate. Within the host, these sequences are found to be physically linked to each other and linked to the selectable marker. These sequences, then, probably exist as a single, large, concatameric structure. We can estimate from cotransformation frequencies that the size of this structure, which we have called the pekelasome, is on the average about 1000 kb, or about 0.1% of a mammalian genome. It is not clear from our work whether pekelasomes are al-

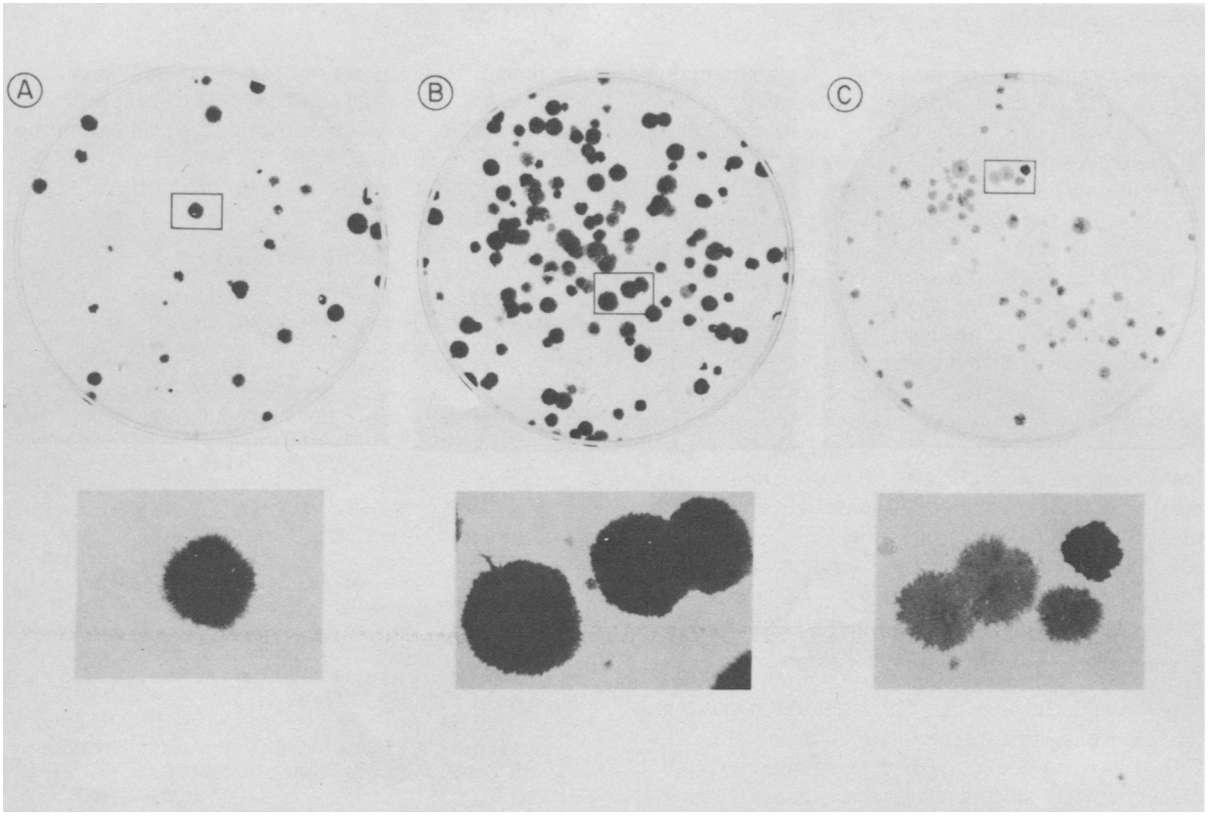

Figure 7. Cells from clone 1a (Table 4) were plated in 100-mm dishes containing different media. After 12–15 days, the plates were washed in PBS, fixed in 50% methanol and 50% acetone for 1–2 min at room temperature, and stained with 10% Giemsa in hot tapwater for 15–30 min. (*A*) HAT medium: 100 cells, 15 days of culture; (*B*) DMEM: 200 cells, 13 days of culture; (*C*) BrdU: 10×10^3 cells, 13 days of culture.

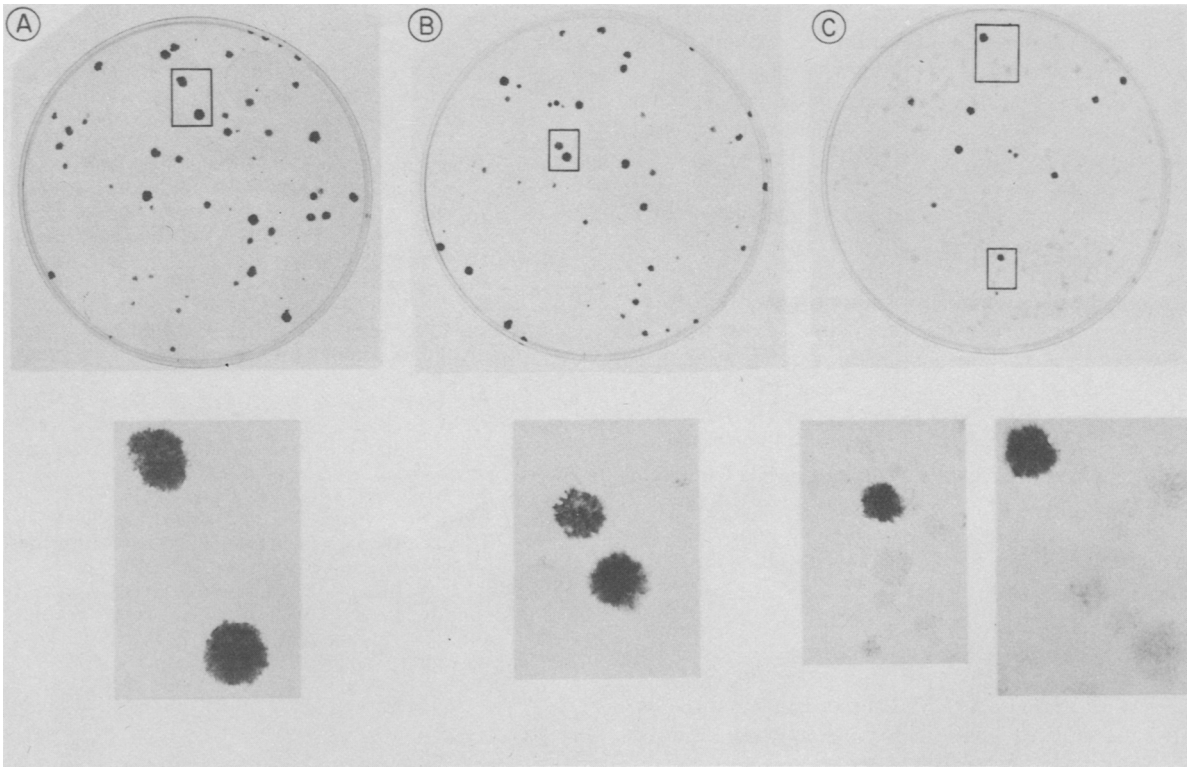

Figure 8. Cells from clone 1b (Table 4) were plated in the indicated media and after 10–12 days stained with Giemsa, as described in the legend to Fig. 7. (*A*) HAT medium: 100 cells, 12 days of culture; (*B*) DMEM: 100 cells, 10 days of culture; (*C*) BrdU: 50×10^3 cells, 10 days of culture.

ways integrated into host chromosomes. In situ hybridization of metaphase chromosome spreads, recently reported by Robins et al. (1981), indicate that pekelasomes are usually, if not always, chromosome-associated in stable transformants derived from a tk^- rat cell line. We presume, however, that in its early stages of formation, the pekelasome may reside extrachromosomally and, as a result, may not be stably inherited by daughter cells. The mechanisms of concatamer formation are unknown. The ligation of linear DNA fragments could be mediated by repair enzymes, either present constitutively or induced by the presence of DNA molecules with free ends, or mediated by a more specific system with another physiological function. Study of the DNA sequence between two previously unlinked fragments, for example, might shed some light on the evolution of the vertebrate chromosome.

However the pekelasome is structured, it appears from our studies using the chicken *tk* and HSV-1 *tk* genes that certain genes will be expressed with high probability whenever they are present in the transformed host. This conclusion does not, however, seem to apply to all genes. For example, when the rabbit β-globin gene has been inserted by cotransformation into Ltk^- cells, both qualitative and quantitative expression varies between independent transformants (Mantei et al. 1979; Wold et al. 1979). We do not yet have sufficient experience with gene transfer systems to decide whether the different behavior of cotransformed genes reflects properties intrinsic to the gene or rather the chance outcomes inherent in the gene transfer process itself.

We can utilize our observations on genetic linkage and the expression of unselected cotransformed markers to facilitate the detection of phenotypic markers in single-copy gene transfer experiments. A tk^- recipient is used, and the transfer is performed in the presence of a cloned *tk* gene under conditions that ensure that at least 50% of transformants for the first marker will also be transformed for *tk*. In such cases, the two phenotypes of the dual transformant will be linked genetically. This method has several possible advantages over transfer of the single phenotype: When the recipient has a high rate of spontaneous mutation for the first phenotype, gene transfer can be distinguished from mutation by, first, the presence of the tk^+ phenotype and, second, by the genetic linkage of the two markers. When the detection of the first phenotype is by screening rather than by selection, one can narrow the population to be screened to those cells that are capable of growth in HAT medium (approximately a 100–1000-fold narrowing). Finally, if there is no selection to maintain the first phenotype, selection in HAT for the tk^+ phenotype will stabilize its inheritance in transformants.

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