Expression of Adenovirus E1a and E1b Gene Products and the *Escherichia coli* XGPRT Gene in KB Cells

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The recombinant plasmid pSV2-gpt, which contains the *Escherichia coli* XGPRT gene under the control of a simian virus 40 early promoter, was modified to contain the type 2 adenovirus (Ad2) *Xho*I-C (0 to 15.5 map units) restriction endonuclease fragment. Plasmid (pLB206) DNA was introduced into human KB cells by Ca^{2+} -mediated DNA transfection, and transformants were selected in medium containing xanthine, aminopterin, and mycophenolic acid, as a consequence of expression of the dominant, selectable XGPRT gene. A series of 13 gpt⁺ cell lines were isolated and tested for their ability to complement Ad5 deletion mutants in E1a (H5d1312) and E1b (H5d1315). Four classes of gpt⁺ KB cell lines were identified, including clones constitutively expressing both E1a and E1b, only E1a, or only E1b or not expressing either E1a or E1b. DNA and RNA filter transfer hybridization analysis substantiated the conclusions that those cell lines capable of complementing viral host range mutants contained the appropriate viral DNA sequences and cytoplasmic polyadenylated RNA species. DNA filter transfer hybridization studies also revealed that the transfected vector DNA was stably integrated into chromosomal DNA in the KB transformants and the number of integrated sites ranged from 1 to 3. The gpt⁺ KB cell line that only expressed E1b gene functions only contained viral E1b gene sequences; those cell lines that expressed neither E1a nor E1b gene function contained only small or no regions of Ad2 DNA. When weaned off the selective medium, transformed KB cell lines stably maintained their inserted DNA in the absence of selective pressure and could easily be adapted to growth in suspension culture.

Exogenous nucleic acid sequences can be readily transferred into mammalian cells (10, 17, 32, 41) by direct selection of those cells which are expressing the gene product(s) encoded by that DNA. Initially, selectable genes such as the herpes simplex virus type 1 thymidine kinase (TK) gene (37, 53), the mouse dihydrofolate reductase gene (30, 52), and the hypoxanthine phosphoribosyl transferase gene (49, 55) were used to complement genetically deficient cells. Later studies (23, 54) demonstrated that nonselectable genes, such as rabbit or human β -globin, could be introduced into eucaryotic cells when they were covalently linked or simply mixed with a selectable gene. Grodzicker, Klesig, and co-workers (18, 28) then cotransferred nonselectable restriction endonuclease fragments of type 2 adenovirus (Ad2) DNA and the herpes simplex virus type 1 TK gene into TK⁻ human cells (termed 143 cells). Thus, they obtained permissive cell lines which resembled 293 cells (16), an Ad5-transformed human embryo kidney cell line, in that the transformed cells constitutively expressed adenovirus E1a and

E1b gene products and were able to complement host range mutants with defects in these regions.

Although cotransfer of several unselected genes was accomplished with the TK-cotransfection system, the technique is limited to recipient cells which are TK⁻ and appears to depend on the continuous presence of selective pressure (HAT [hypoxanthine, aminopterin, thymidine] medium) to prevent loss of the TK and cotransfected genes (1, 53). Recent studies with dominant selectable genes, such as the procaryotic genes for xanthine-guanine phosphoribosyl transferase (XGPRT) and aminoglycoside 3'-phosphotransferase, have indicated the feasibility of cotransfecting eucaryotic cells without defined genetic lesions, using plasmid vectors which contain a eucaryotic viral promoter, a dominant selectable gene, and an unselected gene (8, 33-35).

In the present paper, the recombinant plasmid pSV2-gpt (33) was modified to contain the Ad2 *Xho*I-C restriction endonuclease fragment (0 to 15.5 map units) and used to transfect human KB cells. Initial expression of the XGPRT gene

allowed direct selection for transformed cells by their ability to grow in culture medium containing xanthine, aminopterin, and mycophenolic acid. Four types of cloned stable KB cell lines were generated with XGPRT transfection. These cell lines included those which constitutively expressed both E1a and E1b gene products, only E1a gene products, or only E1b gene products.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of human KB cells and human 293 cells (16) were grown in Dulbecco modified Eagle medium (11) supplemented with 10% calf serum, streptomycin (100 $\mu\text{g/ml}$), and penicillin (100 $\mu\text{g/ml}$). XGPRT-transformed KB cells were cultivated in selective medium consisting of Ham's F12 medium (GIBCO), supplemented with 10% calf serum, mycophenolic acid (50 $\mu\text{g/ml}$), xanthine (250 $\mu\text{g/ml}$), and aminopterin (2 $\mu\text{g/ml}$).

Viral stocks of plaque-purified Ad5 were prepared in KB cell suspension cultures as previously described (12). The viral host range mutants H5d/312 (deletion, 1.5 to 4.5 map units) and H5d/315 (deletion, 3.5 to 8.5 map units) were provided by T. Shenk (24) and were propagated on 293 cell monolayers.

To determine viral yields and viral single-step growth curves from the various gpt⁺ KB cell lines, confluent monolayers were infected at a multiplicity of infection of between 1 and 3 focus-forming units per cell, and the cells were harvested after incubation at 37°C. Progeny virus was released from the infected cells by repeated cycles of freezing and thawing. Viral yields were assayed by means of plaque assay (29, 56) and indirect immunofluorescence assay (fluorescent focus assay), as previously described (12).

Construction of recombinant plasmids. The steps involved in the modification of the chimeric plasmid pSV2-gpt (provided by R. C. Mulligan and P. Berg) are diagrammed and described in Fig. 1a. Both the single-stranded recombinant phage fd103 (21) and plasmid pIF42 were kindly supplied by D. Figurski. Restriction endonucleases *Bam*HI, *Pst*I, *Eco*RI, and *Xho*I and T4 DNA ligase were obtained from New England Biolabs and were used as recommended. Ligation reactions and transformation of *Escherichia coli* K-12 HB101 cells were carried out as previously described (7). Transformants were plated on selective LB agar plates containing either penicillin G (150 $\mu\text{g/ml}$) or chloramphenicol (50 $\mu\text{g/ml}$). Preparative quantities of supercoiled plasmid DNAs were obtained according to the method of Clewell and Helinski (6), except that chloramphenicol-resistant strains were amplified with 250 μg of spectinomycin sulfate (Sigma Chemical Co.) per ml.

The Ad2 *Xho*I-C fragment (0 to 15.5 map units) was purified by two rounds of electrophoresis in low-melting-point agarose (Bethesda Research Laboratories). Without further modification, this DNA was ligated for 16 h at 15°C with a 20-fold molar excess of *Bam*HI linkers (Collaborative Research) which had previously been reacted with T4 polynucleotide kinase. After phenol extractions and precipitation in ethanol, the ligated DNAs were digested with *Bam*HI to activate the linkers and with *Xho*I to destroy viral

DNA dimers. Unligated linker DNA was separated from viral DNA by gel electrophoresis, and the viral DNA was ligated to pLB204 plasmid DNA which had been cleaved with *Xho*I and partially digested with *Bam*HI. HB101 cells were transformed and chloramphenicol-resistant colonies were selected; they were subsequently screened for the viral DNA insert by filter colony hybridization (2), using nick-translated ³²P-labeled *Xho*I-C DNA (42) as the probe. The physical map of this recombinant plasmid, pLB206, is shown in Fig. 1b.

DNA transfections. KB cells were plated at a density of 1.5×10^6 cells per 10-cm dish and were grown for 18 h in Dulbecco modified Eagle medium containing 10% calf serum. With 20 μg of supercoiled plasmid DNA, but excluding salmon sperm DNA as the carrier (34), a calcium phosphate precipitate was formed (17) and added to the cells. After 4 h at 37°C, the cell monolayer was incubated with 15% glycerol in phosphate-buffered saline (15), washed with phosphate-buffered saline, trypsinized, and replated at various densities in Dulbecco modified Eagle medium containing 10% calf serum. Seventy-two hours after replating, the transfected cell monolayers were treated with the selective medium as previously described (34). Approximately 20 to 30 days after the initial selection was initiated, colonies were visible and large enough to be isolated with cloning cylinders. The XGPRT transfection efficiency was determined by fixing the colonies in formaldehyde and staining with 10% Giemsa. gpt⁺ KB cell lines were eventually withdrawn from the selective medium sequentially, using mixtures of selective and nonselective media at ratios of 1:1, 0.5:1, and 0.25:1 and finally nonselective medium alone, at successive cell passages.

Immunofluorescent staining. gpt⁺ KB cells grown on 1.5-cm glass cover slips were fixed with 10% acetone for 10 min at room temperature and then rinsed extensively with phosphate-buffered saline. The fixed monolayer was then treated for 45 min at room temperature with 0.4 μl of antiserum (diluted 1:100) from hamsters bearing tumors induced by Ad2⁺ ND4-transformed cells. After several washes with phosphate-buffered saline, the cells were counterstained with 0.4 ml of a fluorescein-rhodamine mixture for 45 min. Stained cells were visualized and photographed, using an Olympus microscope with a UV light source.

Nucleic acid preparations. Cellular DNA was extracted from approximately 10^8 gpt⁺ KB cells as previously described by Pellicer et al. (40). Low-molecular-weight DNA was extracted from 10^8 gpt⁺ KB cells as previously described by Hirt (22). The Hirt supernatant was phenol extracted once, ethanol precipitated, and suspended in the appropriate restriction endonuclease reaction buffer.

Cytoplasmic RNA was isolated from approximately 10^8 gpt⁺ KB cells by first isolating cell nuclei, using 1% Nonidet P-40, followed by several extractions of the supernatant fluids with phenol-chloroform-isoamyl alcohol (50:48:2) and precipitation of the nucleic acid with ethanol (13). Polyadenylated [poly(A)⁺] RNA was isolated by chromatography on oligodeoxythymidylic acid-cellulose, type 3 (Collaborative Research).

Nucleic acid hybridizations. DNA from transformed cells was cleaved with the appropriate restriction endonucleases and electrophoresed in 0.6% agarose gels in Tris-borate buffer (90 mM Tris, pH 8.0, 90 mM

FIG. 1. Diagrammatic representation of the cloning protocol used to obtain the pSV2-gpt modified plasmid, pLB204 (a). pSV2-gpt plasmid DNA was cleaved with *Bam*HI and ligated to a 1.2-kbp DNA fragment encoding the chloramphenicol transacylation gene, which was isolated by *Bam*HI digestion of phage fd103 replicative form (RF) DNA.

boric acid, 2.5 mM EDTA) at 2.5 V/cm for 16 h. After electrophoresis, DNAs in the gels were depurinated, denatured, transferred onto nitrocellulose filters (Schleicher & Schuell) (26, 48, 51), and annealed with the appropriate ^{32} P-labeled nick-translated DNA fragment or plasmid.

Poly(A)⁺ mRNA species (1 to 5 μ g) were dissolved in a buffer containing 50% formamide (Bethesda Research Laboratories), 2.2 M formaldehyde, and 1 $\times$ running buffer (20 mM morpholinepropanesulfonic acid, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) and were fractionated on 0.8% agarose gels containing 2.2 M formaldehyde (9). After electrophoresis, the RNAs were transferred to nitrocellulose filters (50) and hybridized with ^{32} P-labeled adenovirus E1a (0 to 4.5 map units)- or E1b (5 to 10.1 map units)-specific DNA probes.

RESULTS

Construction of pLB206. The steps involved in the construction of the chimeric plasmid pLB206 are illustrated in Fig. 1. The initial goal was to incorporate another selectable gene into the plasmid, pSV2-gpt (33), and in so doing generate a series of unique restriction endonuclease sites. The procedure was accomplished by using the replicative form of the single-stranded phage fd103 (21) as a donor for the chloramphenicol resistance gene, and in this manner pLB201 was obtained. This plasmid was further modified to contain a unique *Xho*I restriction endonuclease site, so that the Ad2 *Xho*I-C fragment (0 to 15.5 map units) could be ligated into it. The viral fragment was modified to contain a unique *Bam*HI site at its 5'-terminal end and was thus suitable for ligation into pLB204. The resulting plasmid, pLB206, now contained the *E. coli* XGPRT gene fused to the simian virus 40 early promoter and the adenovirus E1a and E1b genes with their own transcriptional regulatory sequences.

DNA-mediated transformation of cells. Thirteen clones were obtained and expanded into cell lines which were used for the experiments to be described. Mulligan and Berg previously showed (33-35), using this type of vector, that 2 to 25 or 10 to 125 XGPRT-expressing cell lines

could be isolated per 10⁵ monkey or mouse cells, respectively. In the studies to be described, the frequency was found to be 1 to 2 per 10⁶ KB cells, and in an analogous study 200 to 280 per 10⁶ CREF cells (a cloned rat embryo fibroblast cell line; 14) was the frequency found. It should be noted that initially selected gpt⁺ cell lines could not be plated directly in nonselective medium. Owing to the accumulation of aminopterin in the cells during the selective process, it was necessary to wean the cloned cells by propagating them in selective medium containing progressively smaller doses of aminopterin. After additional passages the stability of the gpt⁺ KB cell lines was investigated by growing them under nonselective conditions. After 15 passages in Dulbecco modified Eagle medium supplemented with 10% calf serum, the cloned cells displayed a plating efficiency of approximately 1.0 when equal numbers of cells were plated in either selective or nonselective media. Moreover, as will be described, these cells stably maintained the transformed genes for many subsequent passages.

Initial characterization of the gpt⁺ KB clones. Data in the previous section demonstrated that modified pSV2 vector DNA could be used to transform various cell lines from the gpt⁻ to the gpt⁺ phenotype. To determine whether or not any of the gpt⁺ KB cell lines expressed adenovirus E1a or E1b functions or both after transformation with pLB206, they were tested to determine whether they could complement deletion mutants defective in either E1a or E1b functions.

Replicate monolayers of each cell line were infected with wild-type virus, H5d/312, a host range deletion mutant defective in E1a function (24), or H5d/315, a host range deletion mutant defective in E1b function (24). One culture was used as an uninfected control and another was used to count the number of cells in the monolayer. Viral infections were carried out at a multiplicity of infection of 1 to 3 focus-forming units per cell, and the cells were harvested after 3 days of incubation at 37°C, at which time the

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After the transformation of *E. coli* HB101 cells, chloramphenicol- and ampicillin-resistant clones were isolated. The resulting plasmid, pLB200, was obtained and further modified by cleaving it and fd103 RF DNAs with *Eco*RI and *Pst*I. After ligation and transformation, the isolated plasmid pLB201 contained unique *Pst*I and *Eco*RI enzyme sites. To introduce an *Xho*I site into this plasmid, a 1.2-kbp fragment was obtained by digesting pIF42 DNA with *Pst*I and ligating it with pLB201 DNA cleaved with *Pst*I. pLB204 was obtained by selecting for chloramphenicol-resistant colonies which were ampicillin sensitive and contained a unique *Xho*I restriction endonuclease site. For all diagrams the hatched box area represents *E. coli* XGPRT gene sequences, the solid black boxed sequences represent the β -lactamase gene from plasmid pBR322, a stippled box represents the chloramphenicol transacetylation gene from fd103, and all remaining fd103 DNA sequences are represented by a bold black line. The open box area present in pLB204 represents sequences from pIF42 that contain a unique *Xho*I cleavage site. (b) Structure of plasmid pLB206. The construction of this plasmid is described in detail in the text. The Ad2 *Xho*I-C DNA fragment (0 to 15.5 map units) with a *Bam*HI linker at 0 map units is shown as a solid black line at the top and has been substituted for those sequences between the dotted lines. All remaining DNA sequences and symbols have been described for (a).

TABLE 1. Growth of H5d/312 (E1a), H5d/315 (E1b), and H5 wild-type (WT) in representative gpt⁺ KB cell lines and in the parental KB line

KB cell line	Viral yield (FFU/cell) ^a			KB cell line yield of mutant/yield of WT (%)		Yield of mutants in gpt ⁺ KB cell/yield of mutants in parental KB cell ^b	
	312	315	WT	312	315	312	315
gpt ⁺ 2 (1) ^c	0.11	0.26	195	0.058	0.13	0.41	1.4
gpt ⁺ 3 (1)	0.044	13	1,609	0.0027	0.81	0.02	8.7
gpt ⁺ 6 (2)	25	38	360	7.0	11.0	21	111
gpt ⁺ 7 (2)	0.051	0.94	443	0.012	0.21	0.036	2.1
gpt ⁺ 8 (1)	77	0.13	500	16	0.027	105	0.29
gpt ⁺ 16 (2)	239	44	1,439	17	3.1	52	31
gpt ⁺ 18 (1)	0.097	19	137	0.071	14	0.51	151
KB (1)	0.98	0.65	707	0.14	0.093		
KB (2)	0.48	0.14	142	0.33	0.099		

^a FFU, Focus-forming units.^b Ratios have been normalized to take account of the fact that the H5 WT yield varied among the yields from gpt⁺ cell lines and the parental KB cell line. The ratios are expressed as fold increase.^c Numbers in parentheses refer to the corresponding assay in parental KB cells. The normalization of mutant yield, referred to in footnote *b*, was performed on data from the appropriate assay.

amount of cytopathology and the acidity of the supernatant medium were noted. Viral yields were titrated on human 293 cells, and the yield per infected KB cell was calculated. Representative results (Table 1) showed that four of the cell lines tested (no. 6, 8, 16, and 18) displayed an enhanced ability to support replication of one or both deletion mutants when compared with the KB control cells. Other cell lines, for example, no. 7, did not allow replication of the mutants and were shown to lack adenovirus DNA sequences when examined by hybridization blot analysis (see below).

Viral replicative cycles in selected gpt⁺ KB clones. The initial screening suggested that at least four cell lines allowed some replication of H5d/312 or H5d/315 or both. However, to prove this point required a careful examination of the replicative cell cycles of each virus in these cell lines, since neither virus is absolutely defective in either the parental KB cell line (Fig. 2) or HeLa cells (46) and the degree of defectiveness is inversely proportional to the multiplicity of infection (46). Three of the cell lines were examined, and the replicative cycles of H5WT, H5d/312, and H5d/315 were determined and compared with those obtained with the fully permissive 293 cell line and the restrictive KB cell line.

Replicate monolayers of the cell lines were infected with the appropriate viruses at 5 focus-forming units per cell, individual samples were obtained at intervals, and viral yields were determined with 293 cells. As shown previously (46), the replications of H5d/312 and H5d/315 were indistinguishable from that of H5WT on 293 cells (data not shown). In contrast, both deletion mutants replicated poorly on KB cells

(Fig. 2A). In KB cells, however, there was a significant increase above eclipse values in the case of H5d/312, although the eclipse period extended for a longer period than in wild-type replication. KB cell line no. 16 (Fig. 2B) appeared to support replication similar to that in 293 cells since both deletion mutants replicated to high titer and showed an eclipse period similar to that for H5WT in the same clone. These data indicate that infected line 16 cells expressed sufficient adenovirus early functions to complement the defects in E1a and E1b of the two deletion mutants. It will be shown below that these cells contain adenovirus DNA sequences and express mRNA from these regions. The gpt⁺ KB cell line 18 appeared to complement E1b functions only, since H5d/312 failed to replicate more efficiently than in KB cells, whereas H5d/315 reached a titer almost equivalent to that of wild-type virus. However, the eclipse period was longer. As will be shown, cell line 18 lacks E1a DNA sequences and E1a mRNAs.

KB cell line 8, on the initial screen, was classified as a line which expressed E1a functions; however, the absolute level of complementation was low (Fig. 2D) compared with H5d/312 infection of 293 cells. Subsequent data suggested that cell line 8 is a relatively poor expresser of E1a DNA sequences when compared with both cell lines 6 and 16 as well as with 293 cells. It is suspected that the lower level of expression and complementation reflects an intrinsic property of the cell line rather than a mixture of expressing and nonexpressing cells, since immunofluorescence data suggest that all cells contain E1 polypeptides (see below).

Immunofluorescent studies with representative

FIG. 2. Viral multiplication in the parental KB cell line (A) and *gpt*⁺ KB cell lines 16 (B), 18 (C), and 8 (D). Monolayer dishes of the various cell lines were infected with 5 PFU of H5d1312 (●), H5d1315 (□), or H5WT (Δ) per cell at 37°C. Samples were taken at various times, frozen and thawed to release intracellular virus, and titrated for infectivity at 37°C by the fluorescent focus assay, using 293 cells. wt, Wild type; P.I., postinfection.

***gpt*⁺ KB clones.** The preceding virological data imply that newly introduced adenovirus E1 sequences can function properly in some of the *gpt*⁺ KB cell lines. Further evidence that these viral polypeptides are produced may be obtained by indirect immunofluorescence, using antisera specific for antigens encoded by the E1 region in transformed cells.

Antiserum from tumor-bearing hamsters was reacted with *gpt*⁺ KB cell lines 6, 7, 8, 16, and 18, the parental KB line, and 293 cells. All of the E1-expressing cell lines displayed immunofluorescent cells, whereas the nonexpressing cell line 7 and the uninfected KB line did not. KB cell line 8 displayed a diffuse nuclear immunofluorescence, whereas KB cell lines 6, 16, and 18 showed a more particulate staining. Preliminary immunoprecipitation data showed that the antiserum used reacted with the major E1b 58,000-dalton polypeptide expressed in 293 cells (data not shown), but presumably it also reacted with E1a products since cell line 8 showed immunofluorescence. These data, although incomplete as to the specificity of the antigen-antibody reactions, clearly demonstrated that cells capa-

ble of complementation constitutively expressed some E1 functions.

The indirect immunofluorescence technique was also used to determine the stability of the expression of the adenovirus sequences. KB cell clone 16 was examined in detail, first in monolayer cultures and then after its adaptation to spinner culture. No diminution in the percentage of immunofluorescent cells was noted after multiple passage in spinner culture. These data, coupled with the stability of XGPRT expression, the DNA sequence arrangements, and adenovirus mRNA expression, suggest that once the cloned cell lines were established, little change occurred in their phenotype.

Other phenotypic characteristics of the *gpt*⁺ KB cell lines. For many biochemical and genetic manipulations, it is valuable to have cell lines that grow well in either monolayer or spinner culture, a characteristic of the parental KB line. *gpt*⁺ KB cell clones 6, 8, 16, and 18 readily grew in suspension culture and have been maintained as spinner lines for periods of up to 2 weeks with no loss of growth potential.

All cell lines, with the exception of 6, were

suitable for plaque assays. For example, Ad5 wild-type virus had a plaquing efficiency on cell line 18 similar to that on the parental KB cell line. Moreover, since 18 complemented H5d/315, hr 6, a group II host range mutant (20), was examined and shown to develop plaques on these cells as well as on 293 cells at a frequency several orders of magnitude greater than on KB cells. Conversely, hr 1, a group I host range mutant, did not produce plaques on monolayers of cell line 18 (data not shown).

Adenovirus sequences in gpt⁺ KB cell lines. Complementation analysis revealed that of the 13 gpt⁺ KB cell lines isolated, only 4 contained some portion of the adenovirus E1 transcription unit that could be expressed in functional form. To determine the extent of the viral sequences in these cell lines, DNA blot transfer hybridizations (48) were performed. With this method the following were determined: (i) whether E1a or E1b viral gene sequences, or both, were present; (ii) whether the vector DNA existed in an extra-chromosomal or integrated form; and (iii) the relative copy number of the integrated DNA(s) and the number of integration sites.

*Xba*I and *Bam*HI cleaves plasmid pLB206 at three unique sites (one within the Ad2 genome) to generate DNA fragments of 7.85 and 1.35 kilobase pairs (Kbp) (Fig. 1b) when probed with ³²P-labeled Ad2 *Xho*I-C (0 to 15.5 map units) DNA. The 1.35-kbp fragment represents adenovirus sequences from map positions 0 to 3.85, viral E1a gene sequences. The 7.85-kbp segment contains adenovirus sequences from map positions 3.85 to 15.5, representing E1b and a portion of E1a and the adjacent pBR322 and XGPRT DNA sequences. The blot analysis is shown in Fig. 3A and reveals that KB cell lines 8 and 16 contained E1a sequences, which correlated with their ability to complement the E1a host range mutants. Since initiation of E1a transcription occurs at 1.3 map units (3, 38), it was conceivable that some additional cell lines could contain the E1a gene if integration had occurred in a way to alter the *Bam*HI site at the 5'-terminal end of the *Xho*I-C fragment. Using only the restriction endonuclease *Bam*HI, it was determined that cell line 6 did in fact contain an alteration at the 5' terminus of the viral gene (data not shown). Since the cell line was capable of complementing H5d/312, a functional E1a gene must be present.

Further analysis of gpt⁺ KB cell line 6 and 16 cellular DNA, with the restriction enzyme *Sma*I (Fig. 3B), revealed that E1a and E1b gene sequences were not contiguous in cell line 6, but were covalently linked in cell line 16. This finding is consistent with the evidence (shown below) that in cell line 6 cells E1a and E1b transcription might initiate at cellular promoters

in the flanking cellular sequences, since poly(A)⁺ mRNA species were larger than would be predicted if proper initiation occurred.

Restriction endonuclease *Sac*I cleaves pLB206 DNA at three sites within the Ad2 DNA to generate fragments of 6.72, 1.92, and 1.86 kbp when probed only with adenovirus sequences. The 1.86- and 1.92-kbp fragments extend from 5.0 to 10.1 and 10.1 to 15.4 map units, respectively (on the viral genome), and thus represent portions of the viral E1b region. Cell lines 6, 16, and 18 contained these diagnostic bands, whereas cell line 8 lacked these fragments (Fig. 3C). These data are consistent with the evidence previously shown that cell lines 6, 16, and 18 express E1b functions and that cell line 8 only produces E1a proteins.

To determine whether any adenovirus sequences had been cotransferred with the XGPRT gene in gpt⁺ cell lines in which viral mRNA could not be detected (see below), high-molecular-weight DNA was isolated and cleaved with *Sac*I or *Xba*I and *Bam*HI endonucleases, and cotransfer frequency was determined with viral DNA as a probe for hybridization. Of the 13 cell lines tested, 5 were devoid of any viral DNA sequences (data not shown). Three cell lines did show the presence of the 1.86-kbp fragment from the E1b gene. These cell lines complemented H5d/315, but to a much lower degree than did cell line 18; the possibility is being investigated that a functional E1a gene product encoded by H5d/315 could be acting in *trans* to induce expression of the resident integrated E1b DNA sequence. The *Xba*I-*Bam*I 1.35-kbp E1a-specific DNA fragment could only be detected in gpt⁺ KB cell lines 8 and 16, whereas one additional cell line contained some portion of the E1a gene sequences but was unable to complement H5d/312.

Several of the cell lines known to express E1a or E1b viral gene products or both were further studied to determine the number of integration sites and the relative vector DNA copy number. With *Eco*RI restriction endonuclease, which cleaves the vector DNA at one site, and pLB206 DNA as the probe, it was shown (Fig. 3D) that most of the cell lines contained one to three integration sites in the chromosomal DNA. The *Eco*RI site present in pLB206 is contained within the chloramphenicol resistance gene; therefore, the number of bands present for any given cell line represents a minimum number of integration sites, since the integration event could have resulted in the loss of this enzyme site. These data also suggest that each cell line contained one to three copies of the vector DNA when compared with the intensity of the marker DNA band, which consisted of five copies.

These observations confirm previous findings

FIG. 3. Analysis of adenovirus E1A and E1B DNA sequences in gpt⁺ KB cell lines. In (A), (B), (C), and (D), 10 μ g of high-molecular-weight DNA from each cell line indicated above each lane was cleaved with the restriction endonuclease(s) indicated above the panel. The digested DNAs were fractionated by electrophoresis in 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized to nick-translated Ad2 XhoI-C [0 to 15.5 map units (m.u.)] (A, C), Ad2 HpaI-E [0 to 4.5 m.u.] (B), or pLB206 (D) DNAs. The right lane marked M in each panel is a reconstruction containing 10 μ g of DNA from uninfected human KB cell and one genome equivalent of pLB206 DNA per diploid cell genome. The sizes of the pLB206 DNA fragments generated by cleavage with the appropriate restriction endonuclease(s) are indicated on the right.

(8, 34), which showed that similar transfecting vectors produced transformed cell lines with few integration sites and low copy numbers. Vector DNA could not be isolated as autonomously replicating forms in these cells.

Virus-specific mRNAs in gpt⁺ KB cell lines. It was shown above that several of the gpt⁺ KB cell lines had the ability to complement viral host range mutants having defects in E1a or E1b genes. DNA blot hybridization analysis revealed

FIG. 4. Expression of adenovirus E1a- and E1b-specific RNAs in gpt⁺ KB cell lines. Five micrograms of cytoplasmic poly(A)⁺ RNAs from gpt⁺ KB cell lines 6, 8, 16, and 18 and from 293 cells was denatured and electrophoresed in 1% agarose gels containing 2.2 M formaldehyde as described in the text. After the transfer to nitrocellulose filters, RNAs were hybridized with nick-translated E1a [0 to 4.5 map units (m.u.) (A)] or E1b [5 to 10.1 m.u. (B)] DNAs. RNA from the lanes marked H5Wt-infected KB cells were isolated from these cells at 20 h postinfection at 37°C in the presence of 50 mM hydroxyurea to allow accumulation of the early viral transcripts. Only 1 µg of cytoplasmic poly(A)⁺ RNA was used in each lane. The sizes of the RNA species produced in these productively infected cells are indicated on the right.

that variable portions of the E1 region of the Ad2 genome were deleted during the integration process, which could explain the different classes of cell lines generated.

To determine whether the sequences present in these cell lines were transcribed and processed into functional viral messages, cytoplasmic poly(A)⁺ RNA was isolated, and the relative size and number of adenovirus-specific mRNA species were determined by agarose-formaldehyde gel electrophoresis and blot transfer hybridization, using either E1a (0 to 4.5 map units)- or E1b (5 to 10.1 map units)-specific probes (9, 50). The autoradiography patterns obtained by this analysis, using gpt⁺ KB cell lines 6, 8, 16, and 18, are shown in Fig. 4.

During the early phases of an adenovirus productive infection, two mRNA species are transcribed from the E1a genes (3, 5, 27). In Ad2-infected KB cells, or uninfected 293 cells, these two RNA species appear to migrate as a doublet when electrophoresed in agarose gels, with sizes of 13S (1.03 kbp) and 12S (0.91 kbp). KB cell lines 6, 8, and 16 produced viral mRNAs from this region, as predicted, since they complemented viruses with deletions in the E1a

region (Fig. 4A). However, only cell lines 8 and 16 produced both the 13S and 12S mRNAs, whereas KB line 6 made mRNAs that were slightly larger. Consistent with the fact that cell line 18 was unable to complement *d1312*, no E1a mRNAs were detected.

KB cell line 6 produced two E1a-specific mRNAs of approximately 2.2 and 1.2 kbp in length. Since these cells were shown to contain an alteration of viral sequences around the *Bam*HI site at the 5' terminus of the Ad2 *Xho*I-C fragment, it is conceivable that these messages were initiated at a cellular promoter adjacent to the integrated viral DNA. Alternatively, proper initiation could have occurred at map position 1.3, and improper splicing or termination could have resulted in the larger mRNAs observed. However, inaccurate termination seems less likely since E1b probes did not detect these larger messages.

The E1b region encodes two mRNA species of 23S (2.3 kbp) and 9S (1.09 kbp) sizes during the early phases of the viral replicative cycle (3, 5, 27). When cytoplasmic poly(A)⁺ RNAs from cell lines 6, 8, 16, and 18 were probed with ³²P-labeled E1b (5 to 10.5 map units) DNA, all four

lines showed the presence of virus-specific mRNAs (Fig. 4B). KB cell lines 6, 16, and 18 produced both the 23S and 9S messages, which is in accord with the ability of these cells to complement the E1b viral host range mutants. A larger mRNA, of approximately 2.4 kbp, was also present in cell lines 6 and 18, but the nature of this RNA species in terms of its origin and function is unknown.

Cell line 8 was previously shown to have the ability to complement *dl312*, but not *dl315*, and was therefore considered to be only an E1a-expressing cell line. DNA hybridization blot analysis confirmed the biological observation by revealing a major deletion in the E1b sequences. The RNA data (Fig. 4B) suggest either that proper initiation at 4.5 map units occurred but termination took place in the flanking cellular sequences, or that adjacent cellular promoters provided the sequences necessary to transcribe the remaining E1b sequences. Since the messages produced in this cell line were larger than those made in the productive infection of 293 cells, and did not produce functional proteins, both hypotheses are plausible and are under investigation.

These studies characterizing the poly(A)⁺ RNAs produced in the *gpt*⁺ KB cell lines containing adenovirus DNA sequences also revealed species of RNAs of unusual sizes (Fig. 4). The origin and function of these RNAs are unclear. It should be noted that, whereas this approach to study the mRNA species synthesized is not quantitative, it is apparent that overall production of E1 mRNAs was at least 2- to 5-fold less in the *gpt*⁺ KB cell lines than in 293 cells or 10- to 20-fold less than in Ad5-infected KB cells.

DISCUSSION

The methods developed in recent years (for reviews, see 31 and 44) for introducing nonselectable genes into mammalian cells are particularly suitable for studying eucaryotic and viral gene regulation, viral genetics, and cellular transformation. In this paper it was demonstrated that, by using the *E. coli* XGPRT gene as a dominant selectable marker, mammalian cells can be used for cotransformation assays with a plasmid vector, pLB206, which contains adenovirus DNA sequences encoding E1a and E1b gene functions. Mulligan and Berg (33, 34) showed that this system is amenable to use in a wide variety of mammalian cells, and we have confirmed their findings by transforming both permissive human (KB) and nonpermissive rat (CREF) cell lines, using pLB206 DNA. KB cell lines were further studied for their ability to co-express the nonselectable viral gene functions because these cells (i) yield large quantities of cell-associated

virus, (ii) can grow in suspension culture, (iii) can produce viral plaques at a wide temperature range, and (iv) can be used with normal KB cell lines for comparative studies involving viral host range mutants with defects in the E1 region of the viral genome.

In the experiments described, KB cells were initially selected for their ability to express the *E. coli* XGPRT gene product. Thirteen independent cell lines were isolated and grown to mass culture for the purpose of determining whether they were capable of expressing viral E1a or E1b gene products or both. Complementation analysis with the viral host range mutants H5*dl312* (deletion in E1a) and H5*dl315* (deletion in E1b) (24) revealed that, of the 13 cell lines tested, cell lines 6 and 16 were capable of expressing E1a and E1b gene functions, cell line 8 solely expressed E1a functions, and cell line 18 only expressed E1b functions.

To confirm the presence of integrated virus-specific DNA sequences and the nature of the viral messages in *gpt*⁺ KB cell lines 6, 8, 16, and 18, both DNA and RNA analyses were performed. DNA blot hybridization studies confirmed previous findings (34) that relatively few copies of the vector plasmid were present in an integrated form. It was initially proposed (34) that the low copy numbers observed might be due to the detrimental effects produced by the expression of the selectable marker in the transformed cells. This notion was recently investigated in a cotransformation assay that used both the XGPRT and dihydrofolate reductase genes. Using Chinese hamster ovary cells and progressively increasing concentrations of methotrexate, Ringold et al. (43) demonstrated that XGPRT sequences could be amplified in both gene copy numbers and overall protein production. It is therefore possible that the low copy numbers observed are caused by a low intrinsic efficiency of integration exhibited by the individual cell.

It was consistently observed that the nonpermissive cell lines transfected with pLB206 DNA contained numerous copies of the vector sequences (data not shown), but it has not yet been determined whether this finding correlates with viral or XGPRT gene expression. Gene amplification of transfected DNA sequences occurs after selection for expression (19, 45), and therefore these rodent CREF cell lines are presently being studied for evidence of such a phenomenon.

DNA hybridization studies further confirmed the existence of viral DNA sequences encoding E1a gene functions for *gpt*⁺ KB cell lines 6, 8, and 16 and E1b nucleotide sequences for cell lines 6, 16, and 18, supporting the complementation data. Moreover, the evidence obtained indi-

cated that neither vector nor viral DNA existed in an episomal form.

Hybridization analyses also revealed that those cell lines capable of complementing E1a and E1b host range mutants contained the expected virus-specific poly(A)⁺ mRNA species in their cytoplasm. It should be noted that KB cell line 6 produced E1a and E1b mRNAs of lengths greater than would be predicted if proper initiation, termination, and processing occurred. Since the DNA integration patterns of cell line 6 suggested that a deletion of a portion of the viral sequences existed near the 5' end of the E1a gene, and that the E1a and E1b gene sequences were not physically linked, the mRNA molecules observed would have been produced if the E1a or E1b promoter sequences located at 1.3 or 4.5 map units were deleted and thus initiation of transcription occurred in the flanking cellular sequence; alternatively, aberrant splicing sites, which have been shown to occur in simian virus 40-globin recombinants (36), might have produced the large mRNA species observed. S1 analysis (3), using end-labeled DNA fragments from various regions of the pLB206 plasmid, is presently being used to study these questions.

The finding that KB cell line 18 constitutively expressed viral E1b gene functions in the absence of E1a proteins is consistent with the conclusion drawn from studies made with viruses whose genomes were constructed with mutations in E1a sequences so that the 13S mRNA produced truncated proteins (4, 47). However, our findings are in contrast to conclusions drawn from studies with the host range mutant *d1312* (25), in which all coding sequences necessary for E1a expression are deleted and which does not express any early mRNAs during a nonpermissive infection produced with a low multiplicity of virus (39). Our data may be explained by the possibility that adjacent host cell sequences of cell line 18 supply the initiation site suitable for transcription of the E1b sequences, that integration has so altered the environment of the E1b promoter than it can function independently, or that the E1b promoter naturally serves this function. Moreover, the E1a promoter sequences were shown to be absent. Thus, E1b can be independently expressed in the complete absence of E1a expression.

Like 293 cells, which contain the Ad5 E1a and E1b sequences, the KB cell clones isolated will be useful in the isolation of mutants with defects in genes being constitutively expressed in these host cells. But these KB cell lines which contain and express only E1a or E1b genes are beginning to fulfill another important role in permitting studies that explore functional interactions between gene products from different regions of the viral genome.

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LITERATURE CITED

1. Bacchetti, S., and F. L. Graham. 1977. Transfer of the gene for thymidine kinase to thymidine kinase deficient human cells by means of purified Herpes Simplex viral DNA. *Proc. Natl. Acad. Sci. U.S.A.* 74:1590-1594.
2. Benton, D., and R. W. Davis. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182.
3. Berk, A. J., and P. A. Sharp. 1978. Structure of adenovirus 2 early mRNAs. *Cell* 14:695-711.
4. Carlock, L. R., and N. C. Jones. 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1a mRNA species. *J. Virol.* 40:657-664.
5. Chow, L. T., T. R. Broker, and J. B. Lewis. 1979. Complex splicing patterns of RNA from the early regions of adenovirus 2. *J. Mol. Biol.* 134:265-303.
6. Clewell, D. B., and D. R. Helinski. 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 DNA and protein in *Escherichia coli*. *J. Bacteriol.* 110:1135-1146.
7. Cohen, S. W., and C. Miller. 1970. Nonchromosomal antibiotic resistance bacteria. III. Isolation of the discrete transfer unit of the R-factor RI. *Proc. Natl. Acad. Sci. U.S.A.* 67:310-316.
8. Colbere-Garapin, F., F. Horodniceanu, P. Kourilsky, and A.-C. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* 150:1-14.
9. Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell, Jr. 1981. Transcriptional control in the production of liver-specific mRNAs. *Cell* 23:731-739.
10. Dimitriadis, G. J. 1978. Translation of rabbit globin mRNA introduced by liposomes into mouse lymphocytes. *Nature (London)* 274:923-924.
11. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130:432-437.
12. Ensinger, M., and H. S. Ginsberg. 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. *J. Virol.* 10:328-339.
13. Esche, H., M. Mathews, and J. B. Lewis. 1980. Proteins and messenger RNAs of the transforming region of wild type and mutant adenoviruses. *J. Mol. Biol.* 142:399-417.
14. Fisher, P. B., L. E. Babiss, I. B. Weinstein, and H. S. Ginsberg. 1982. Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). *Proc. Natl. Acad. Sci. U.S.A.* 79:3527-3531.
15. Frost, E., and J. Williams. 1978. Mapping of temperature-sensitive and host-range mutants of adenovirus type 5 by marker rescue. *Virology* 91:39-50.
16. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-72.
17. Graham, F., and A. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
18. Grodzicker, T., and D. F. Klessig. 1980. Expression of unselected adenovirus genes in human cells co-transformed with the HSV-1 tk gene and adenovirus 2 DNA. *Cell* 21:453-463.
19. Hanahan, D., D. Lane, L. Lipsich, M. Wigler, and M. Botchan. 1980. Characteristics of an SV40-plasmid recombinant and its movement into and out of the genome of a murine cell. *Cell* 21:127-139.
20. Harrison, T., F. Graham, and J. Williams. 1977. Host

- range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* 77:319-329.
21. Hermann, R., K. Nergebauer, E. Pirkl, H. Zentgraf, and H. Schaller. 1980. Conversion of bacteriophage fd into an efficient single-stranded DNA vector system. *Mol. Gen. Genet.* 177:231-242.
  22. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
  23. Huttner, K. M., G. Scangos, and F. H. Ruddle. 1979. DNA-mediated gene transfer of a circular plasmid into murine cells. *Proc. Natl. Acad. Sci. U.S.A.* 76:5820-5824.
  24. Jones, N., and T. Shenk. 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 17:683-689.
  25. Jones, N., and T. Shenk. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. U.S.A.* 76:3665-3669.
  26. Ketner, G., and T. Kelly, Jr. 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A.* 73:1102-1106.
  27. Kitchingman, G. R., and H. Westphal. 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. *J. Mol. Biol.* 137:23-48.
  28. Klessig, D. F., M. P. Quinlan, and T. Grodzicker. 1982. Proteins containing only half of the coding information of early region 1b of adenovirus are functional in human cells transformed with the HSV type 1 TK gene and adenovirus type 2 DNA. *J. Virol.* 41:423-434.
  29. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. *J. Virol.* 1:851-867.
  30. Lewis, W. H., P. R. Srinivasan, N. Stokes, and L. Siminovich. 1980. Parameters governing the transfer of the gene for thymidine kinase and dihydrofolate reductase into mouse cells using metaphase chromosomes or DNA. *Somat. Cell Genet.* 6:333-348.
  31. McBride, O. W., and J. L. Peterson. 1980. Chromosome-mediated gene transfer in mammalian cells. *Ann. Rev. Genet.* 14:321-346.
  32. Mueller, C. A., A. Graessmann, and M. Graessmann. 1978. Mapping of early SV40 specific functions by microinjection of different early viral DNA fragments. *Cell* 15:579-585.
  33. Mulligan, R. C., and P. Berg. 1980. Expression of a bacterial gene in mammalian cells. *Science* 209:1422-1427.
  34. Mulligan, R. C., and P. Berg. 1981. Selection of animal cells that express the *Escherichia coli* gene for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* 78:2072-2076.
  35. Mulligan, R. C., and P. Berg. 1981. Factors governing the expression of a bacterial gene in mammalian cells. *Mol. Cell Biol.* 1:449-459.
  36. Mulligan, R. C., R. T. White, and P. Berg. 1980. Formation of B-globin following infection with recombinants containing rabbit B-globin cDNA at different locations of SV40's late region, p. 201-215. *In* W. A. Scott et al. (ed.), 17th Miami Winter Symposium: mobilization and reassembly of genetic information. Academic Press, Inc., New York.
  37. Munyon, W., E. Kraiselburd, D. Davis, and J. Mann. 1971. Transfer of thymidine kinase to thymidine kinaseless L cells by infection with UV-irradiated herpes simplex. *J. Virol.* 7:813-820.
  38. Nevins, J. R. 1980. Definition and mapping of adenovirus 2 nuclear transcription. *Methods Enzymol.* 65:768-785.
  39. Nevins, J. R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1a gene product. *Cell* 26:213-220.
  40. Pellicer, A. O., M. Wigler, R. Axel, and S. Silverstein. 1978. The transfer and stable integration of the HSV thymidine kinase gene into mouse cells. *Cell* 14:133-141.
  41. Rechsteiner, M. 1978. Red cell-mediated microinjection. *Natl. Cancer Inst. Monogr.* 48:57-64.
  42. Rigby, P. W., J. M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
  43. Ringold, G., B. Dieckmann, and F. Lee. 1981. Co-expression and amplification of dihydrofolate reductase cDNA and the *E. coli* XGPR gene in Chinese Hamster Ovary cells. *J. Mol. Appl. Genet.* 1:165-175.
  44. Scangos, G., and F. H. Ruddle. 1981. Mechanisms and applications of DNA-mediated gene transfer in mammalian cells—a review. *Gene* 14:1-10.
  45. Schimke, R. T., R. J. Kaufman, F. W. Alt, and R. F. Kellems. 1978. Gene amplification and drug resistance in cultured murine cells. *Science* 202:1051-1055.
  46. Shenk, T., N. Jones, W. Colby, and D. Fowlkes. 1979. Functional analysis of adenovirus-5 host range deletion mutants defective for transformation of rat embryo cells. *Cold Spring Harbor Symp. Quant. Biol.* 44:367-375.
  47. Solnick, D., and M. A. Anderson. 1982. Transformation-defective adenovirus mutant defective in expression of region 1A but not region 1B. *J. Virol.* 42:106-113.
  48. Southern, E. M. 1975. Detection of specific sequences among DNA fragments by gel electrophoresis. *J. Mol. Biol.* 38:503-517.
  49. Szybalska, E. H., and W. Szybalski. 1962. Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. *Proc. Natl. Acad. Sci. U.S.A.* 48:2026-2034.
  50. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* 77:5201-5205.
  51. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* 76:3683-3687.
  52. Wigler, M., M. Perucho, D. Kurtz, S. Dana, A. Pellicer, R. Axel, and S. Silverstein. 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. *Proc. Natl. Acad. Sci. U.S.A.* 77:3567-3570.
  53. Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, T. Cheng, and R. Axel. 1977. Transfer of purified Herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 11:223-232.
  54. Wigler, M., R. Sweet, G.-K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell* 16:777-785.
  55. Willecke, K., M. Klomfass, R. Mierau, and J. Doehmer. 1979. Intraspecies transfer via total cellular DNA of the gene for hypoxanthine phosphoribosyltransferase into cultured mouse cells. *Mol. Gen. Genet.* 170:179-185.
  56. Williams, J. F. 1970. Enhancement of adenovirus plaque formation of HeLa cells by magnesium chloride. *J. Gen. Virol.* 9:251-255.