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Molecular cloning and cell cycle-specific regulation of a functional human thymidine kinase gene

(gene rescue/DNA-mediated transformation/cell synchronization)

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ABSTRACT **A functional thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) gene has been molecularly cloned from human DNA. The gene was rescued from a genomic library of TK-deficient mouse L cells transformed to the TK^+ phenotype with total HeLa cell DNA. Of 14 overlapping clones, only one contained the intact human TK gene. The cloned recombinant bacteriophage carries a 16-kilobase insert derived entirely from human DNA and is capable of transforming LTK^- cells to TK^+ with an efficiency of 10 TK^+ colonies per ng of DNA per 10^6 cells. Restriction endonuclease mapping shows that the functional human TK gene is at least twice as long as that reported for chicken. A 1.6-kilobase *Xho* I/*Eco*RI fragment was subcloned and found to hybridize to a human mRNA of 1.5 kilobases. When introduced into LTK^- cells, the cloned human TK gene is regulated in the cell cycle-specific manner characteristic of TK^+ mammalian cells. That is, TK activity in synchronized cells increases markedly with the onset of DNA synthesis. The signals governing the S-phase induction of TK activity reside within 16 kilobases of human DNA and are correctly interpreted by mouse cells.**

Thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.27) is an enzyme in the pyrimidine salvage pathway and is intimately associated with DNA synthesis in many cell types. Cellular TK activity is strongly proliferation-dependent, peaking during the S phase of the cell cycle (1) along with other enzymes involved in DNA replication. The induction of TK activity is believed to be governed by an increase in transcription of the TK gene (for a review, see ref. 2). In terminally differentiating cells, such as cardiac muscle cells, TK activity declines to zero as the cells withdraw from the mitotic cycle (3). It is not known whether the mechanism for short-term regulation of TK gene expression in rapidly dividing cells resembles the long-term repression of TK gene transcription in terminally differentiated cells. Identification of transcriptional control regions in the human TK gene requires cloned probes to these sequences. The availability of such probes for the TK gene of herpes simplex virus has led to an understanding of some important transcriptional signals (4). The herpes simplex virus and human TK genes are regulated in very different ways, making a comparison of their transcriptional controls of considerable interest. To facilitate the study of the control of TK gene expression in human cells, I have applied recently developed techniques for the cloning of human genes transferred to mouse cells (5) to isolate a functional human TK gene. The structure and regulation of the cloned gene have been examined.

METHODS

Transformation of LTK^- Cells to TK^+ with HeLa DNA. LTK^- cells were maintained, transformed with total HeLa DNA, and selected in hypoxanthine/aminopterin/thymidine (HAT) medium (15 μg of hypoxanthine, 0.4 μg of aminopterin, 5 μg of thymidine, and 50 μg of glycine per ml) essentially as described by Wigler *et al.* (6). After the TK^+ colonies had been allowed to grow for 21 days, they were picked by the cloning cylinder technique (7) and expanded into mass culture.

Blot Hybridizations. DNA extracted from the TK^+ transformant cell lines (8) was cleaved with restriction endonucleases in the buffer recommended by the vendor (New England BioLabs). Vertical 1% agarose gels (8) were run at 35 V for 12 hr, stained with ethidium bromide (1 μg/ml) and photographed. *Hin*dIII fragments of λ DNA served as size markers. The gels were blotted to nitrocellulose as described by Southern (9). Polyadenylylated cytoplasmic RNA was prepared (10) from logarithmically growing HeLa cells, fractionated under denaturing conditions on a 1% agarose/2.2 M formaldehyde gel at 25 V for 12 hr, and transferred to nitrocellulose in 20× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7) (11). *Escherichia coli* ribosomal RNAs served as size markers. Filters were treated prior to hybridization in 50 mM sodium Pipes/1 M NaCl/0.1% Ficoll/0.1% bovine serum albumin/0.1% polyvinylpyrrolidone/1 mM EDTA/50% formamide containing 100 μg of denatured calf thymus DNA and 100 μg of yeast RNA per ml. Hybridizations were carried out in the same buffer with the addition of hybridization probes labeled to a specific activity of 2×10^8 cpm/μg by nick-translation (12) with [α-^{32}P]dCTP (New England Nuclear). Hybridizations were carried out at 42°C for 24 hr with a probe concentration of 20 ng/ml (4×10^6 cpm/ml). The hybridized filters were washed twice in 2× NaCl/Cit containing 0.5% sarcosyl for a total of 30 min at 37°C, followed by a 1-hr wash in 0.1× NaCl/Cit containing 0.5% sarcosyl at 55°C. Filters were wrapped in Saran Wrap and exposed to Kodak XRP film for 48 hr at −80°C with 1 DuPont Cronex Lightning-Plus intensifying screen.

Construction of a Genomic Library in the Bacteriophage λ Vector EMBL4. The vector was the generous gift of H. Lehrach. DNA (150 μg) from the primary TK^+ transformant cell line LHTK-2 (see Fig. 1) was partially digested with *Mbo* I to an average size of 20 kilobases (kb) and size-fractionated by centrifugation through a 5–20% potassium acetate/10 mM EDTA gradient in a Beckman SW 28 rotor at 25,000 rpm for 7 hr (13). Fractions containing DNA of 15–24 kb were pooled, precipitated with ethanol, and ligated to a 2-fold mass excess of phage arms (13) prepared from *Sal* I/*Bam*HI-cut EMBL4 DNA. Ligation with 40 units of T4 DNA ligase (New England BioLabs) was carried out at 14°C for 12 hr at a total DNA concentration of 250 μg/ml. Ligated DNA was packaged into phage particles *in vitro* (14) and used to infect *E. coli* C. The library was screened by hybridization (15) to ^{32}P-labeled HeLa DNA under the con-

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Abbreviations: TK, thymidine kinase; kb, kilobase(s); HAT, hypoxanthine/aminopterin/thymidine.

ditions described above, except that 2×10^6 cpm/ml was used and the hybridization mixture contained 10% dextran sulfate. Hybridizing clones were picked, plaque-purified, and subsequently amplified for DNA preparation. All cloning experiments were carried out in accordance with current National Institutes of Health guidelines for recombinant DNA research.

Subcloning the 1.6-kb *Xho* I/*Eco*RI Fragment of λTK46 into pUC8 (16). λTK46 DNA cleaved with *Xho* I and *Eco*RI (3 μg) was ligated to 500 ng of *Sal* I/*Eco*RI-digested pUC8 DNA in 50 μl of ligase buffer at 14°C for 1 hr. Competent (17) *E. coli* JM83 were transformed with the ligated DNA and selected on plates containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (20 μg/ml). A white colony was picked and used to prepare plasmid DNA by alkaline lysis (18). The presence of the desired 1.6-kb *Xho* I/*Eco*RI fragment was verified by restriction mapping.

Cell Synchronization, DNA Synthesis Measurement, and TK Assay. LTK^- cells transformed to TK^+ with the cloned human TK gene were seeded at 1×10^6 cells per 75 cm^2 flask in medium containing 10% fetal bovine serum. The cells were allowed to grow for 2 days and then were fed with serum-free medium, which caused them to accumulate in the G_1 phase; 72 hr later, the serum-free medium was replaced with medium containing 10% fetal bovine serum, allowing the cells to progress through the cell cycle as a synchronous population. At each time point after serum addition, duplicate flasks were assayed for DNA synthesis and TK enzyme activity. DNA synthesis was measured by the incorporation of [^{3}H]thymidine into acid-precipitable radioactivity. Cells labeled for 1 hr with 2 μCi of [^{3}H]thymidine (77 Ci/mmol, ICN; 1 Ci = 3.7×10^{10} Bq) per ml were washed once with sterile phosphate-buffered saline (19), harvested by scraping, and pelleted in a clinical centrifuge. The cell pellet was resuspended in 400 μl of phosphate-buffered saline, followed by the addition of 100 μl of 50% trichloroacetic acid. After standing on ice for 10 min, the precipitate was collected on Whatman GF/C filters and washed with 10 ml of 10% trichloroacetic acid and 10 ml of ethanol. The filter was then dried and assayed for radioactivity by liquid scintillation spectrometry. To assay TK enzyme activity (20), a cell pellet was prepared as above and frozen at −20°C. The cell pellet was disrupted by three cycles of freezing and thawing in 50 μl of 20 mM Tris, pH 7.5/150 mM NaCl/2 mM dithiothreitol/50 μM thymidine/10% glycerol. This lysate was clarified by low-speed centrifugation, and 10 μl of the supernatant was added to 100 μl of a reaction mixture containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 40 μM [^{14}C]-thymidine (14 mCi/mmol, ICN), and 500 μg of bovine serum albumin per ml. The reaction was allowed to proceed for 1 hr at 37°C and was terminated by spotting 40 μl of the reaction onto a 24-mm Whatman DE-81 disk. The disks were batch-washed three times in ethanol, dried, and assayed for radioactivity. Protein assays of the cell pellet extracts were done by the Bio-Rad Coomassie brilliant blue assay with bovine serum albumin as a standard (21). The enzyme activity is expressed as nmol of thymidine converted to dTMP per min per mg of protein.

RESULTS

To clone the human TK gene, LTK^- cells were transformed to a TK^+ phenotype by using total HeLa DNA as the TK gene donor. TK^+ transformants were selected by their ability to survive in HAT medium. Three to five TK^+ transformants per 20 μg of HeLa DNA per 10^6 cells were obtained in primary transformation experiments. With ^{32}P-labeled HeLa DNA as a hybridization probe, the primary TK^+ transformant DNAs were examined for the presence of interspersed highly reiterated human sequences, principally members of the *Alu* family (22) (Fig. 1, lanes a and b). Primary transformants are expected to contain many human *Alu* family members, only a few of which will be closely associated with the selectable TK gene. Secondary transformants produced with DNA from the primary transformants as TK gene donors should contain only those *Alu* family members very near the TK gene (Fig. 1, lanes c and d). Both primary transformants and both secondary transformants contained a hybridizing *Sac* I band of 6.4 kb, presumably shared because it is linked to the human TK gene. One of the primary transformants, LHTK-1, harbored more than 20 *Sac* I fragments containing human repetitive sequences (Fig. 1). DNA from the primary transformant LHTK-2 had fewer than 10 hybridizing *Sac* I fragments and was used to construct a representative genomic library in the bacteriophage λ vector EMBL4. Clones recovered from a primary transformant are less likely to be rearranged as a result of integration into the mouse genome. Such "scrambling" of sequences has been reported in the cloning of the hamster adenine phosphoribosyltransferase gene from a secondary transformant (23). Recombinant phage (1.5×10^6) from the LHTK-2 library were screened by hybridization to ^{32}P-labeled HeLa DNA. Forty-six hybridizing clones were picked; of these, 20 were plaque-purified and amplified for small-scale DNA isolation. Approximately 1 μg of DNA from each of the 20 clones was introduced into LTK^- cells, followed by selection in HAT medium. One clone, λTK46, gave rise to a large number of TK^+ colonies. The TK^+-transforming efficiency of λTK46 was measured precisely and found to be 10 TK^+ colonies per ng of DNA per 10^6 cells.

Restriction mapping (Fig. 2) and blot-hybridization experiments showed that the 16-kb insert is derived entirely from human DNA, as evidenced by the hybridization of human repetitive sequences along the full length of the clone and by the absence of any such hybridization to labeled mouse DNA (unpublished data). The 1.6-kb *Xho* I/*Eco*RI fragment from the right-hand portion of the cloned insert was subcloned into the plasmid vector pUC8 and used to probe *Sac* I digests of the two primary transformants and two secondary transformants shown in Fig. 1. In addition, LTK^- DNA, the HeLa DNA that served

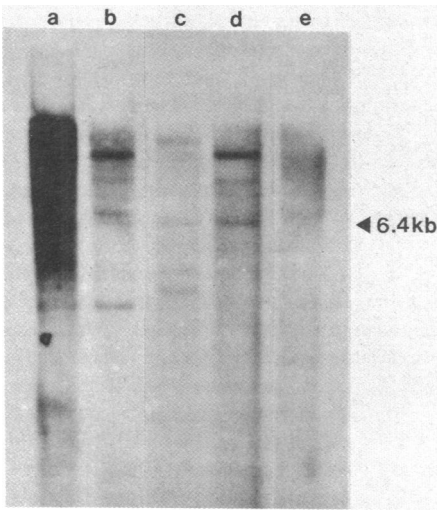

FIG. 1. Southern blot analysis of human sequences in mouse LTK^- cells transformed to TK^+ with HeLa DNA. Each DNA (10 μg) was digested with *Sac* I, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to labeled HeLa DNA as described. Primary transformant cell lines were LHTK-1 (lane a) and LHTK-2 (lane b). Secondary transformants were derived from LHTK-1 (lane c) and LHTK-2 (lane d). LTK^- DNA (lane e) was included as a control.

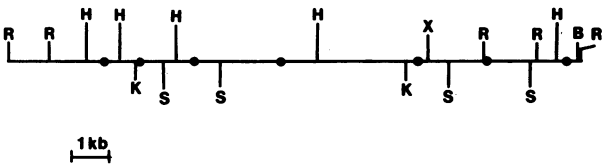

FIG. 2. Restriction endonuclease cleavage map of the 16-kb cloned insert in λTK46. R, *Eco*RI; H, *Hin*dIII; S, *Sac* I; K, *Kpn* I; X, *Xho* I; B, *Bam*HI; ●, human repetitive sequence. The external *Eco*RI sites are present in the bacteriophage vector DNA immediately upstream from the *Bam*HI site into which the human DNA is cloned. A *Bam*HI site was regenerated at the right insert/vector junction but not at the left junction.

as the original TK gene donor, and human peripheral blood lymphocyte DNA were cleaved with *Sac* I and electrophoresed alongside the transformants (Fig. 3). Based on the restriction map of λTK46, a Sac I digest should generate fragments of 6.4 and 2.3 kb, which hybridize to the 1.6-kb *Xho* I/*Eco*RI probe. In the primary and secondary TK^+ transformant cell lines, but not in the parental LTK^- line, the predicted hybridizing bands were observed. Likewise, in the HeLa and lymphocyte DNAs, *Sac* I fragments of the expected size were found, proving that the TK gene carried on λTK46 and in all the TK^+ transformant cell lines is of human origin and suggesting that the 16-kb insert in λTK46 did not undergo rearrangement during cloning.

To delineate the extent of the TK coding and regulatory regions within the 16-kb insert of λTK46, the phage DNA was cleaved with several restriction endonucleases and introduced into LTK^- cells, followed by selection in HAT medium. Digestion with enzymes cleaving within the insert, including *Eco*RI, *Hin*dIII, *Sac* I, *Kpn* I, *Xho* I, and *Pvu* II, abolished TK^+ transforming activity. Cleavage of λTK46 DNA with *Bam*HI, *Bgl* II, and *Sal* I, none of which have a recognition sequence within the 16-kb insert, had no effect on transforming activity.

To determine if the 1.6-kb *Xho* I/*Eco*RI fragment from the genomic clone lies in a transcribed region of the human TK gene, 50 μg of polyadenylylated cytoplasmic RNA prepared from logarithmically growing HeLa cells was examined by blot hybrid-

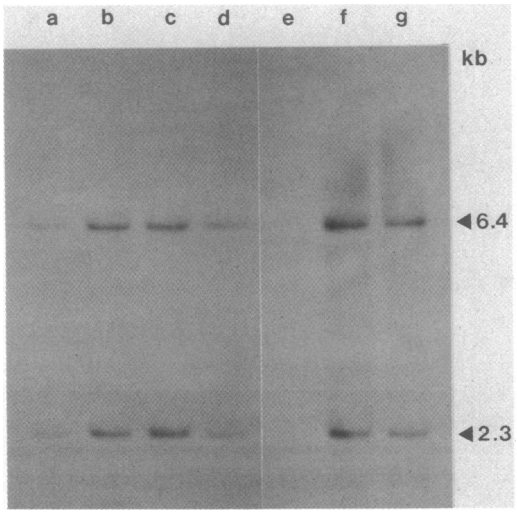

FIG. 3. Primary transformant, secondary transformant, HeLa, and human lymphocyte DNAs cleaved with *Sac* I, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to the 1.6-kb *Xho* I/*Eco*RI fragment from λTK46. Primary transformants were LHTK-1 (lane a) and LHTK-2 (lane b). Secondary transformants were derived from LHTK-1 (lane c) and LHTK-2 (lane d). LTK^- DNA (lane e) was included as a control. Also shown are human DNAs from HeLa cells (lane f) and lymphocytes (lane g).

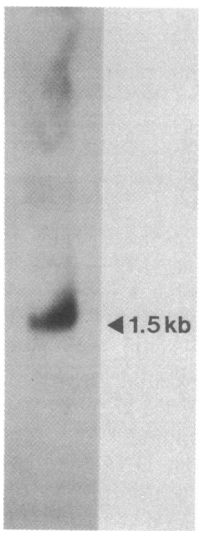

FIG. 4. Cytoplasmic polyadenylylated RNA (50 μg) prepared from logarithmically growing HeLa cells, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to the 1.6-kb *Xho* I/*Eco*RI fragment from λTK46.

ization, The 1.6-kb *Xho* I/*Eco*RI fragment hybridized to an mRNA of 1.5 kb (Fig. 4), which has been shown to be the size of the human TK mRNA (24). In addition, the 1.6-kb *Xho* I/*Eco*RI fragment has been used to isolate an expressible cDNA (25) containing the entire TK coding region (unpublished data). It is clear from these results that the 1.6-kb *Xho* I/*Eco*RI fragment contains some transcribed human TK gene sequences.

It has been reported that TK activity increases markedly during the S phase of the cell cycle and that this cell cycle-specific expression is maintained when the human TK gene is introduced into LTK^- cells by DNA- or metaphase chromosome-mediated transformation (26). The elements controlling the induction of TK activity are, therefore, present on transferred human DNA and are recognized by mouse cells. The number of such controlling elements and their distribution in the human genome remained unknown. By using a synchronized population of LTK^- cells transformed to TK^+ with the human TK gene clone λTK46, it is clear that the coupling of TK activity to DNA replication is dependent only on sequences found within the 16-kb cloned insert (Fig. 5). The cells exhibited a lag time of 12 hr before entering the S phase of the cell cycle, a characteristic of the serum-deprivation method of synchronization. To verify that the incorporation of [^{3}H]thymidine was an accurate measure of DNA synthesis and not just a reflection of the increasing TK activity, parallel flasks of cells were labeled with 2 μCi of [^{3}H]deoxyadenosine and handled as described. The pattern of DNA synthesis observed was identical to that seen with [^{3}H]thymidine (unpublished data).

DISCUSSION

I have cloned a TK gene from mouse LTK^- cells transformed to TK^+ with human DNA. The cloned gene and flanking regions are of exclusively human origin and are colinear with human DNA from both HeLa cells and lymphocytes. Restriction enzyme mapping of cleavage sites that abolish the TK^+-transforming activity of λTK46 reveals that the minimum-length fragment containing the functional TK gene is 4.2 kb. That is, the 4.2-kb fragment of λTK46 extending rightward from the rightmost *Kpn* I site to the *Hin*dIII site contains at least one recognition sequence for each restriction endonuclease that inactivates the gene. However, the possibility that the gene is as

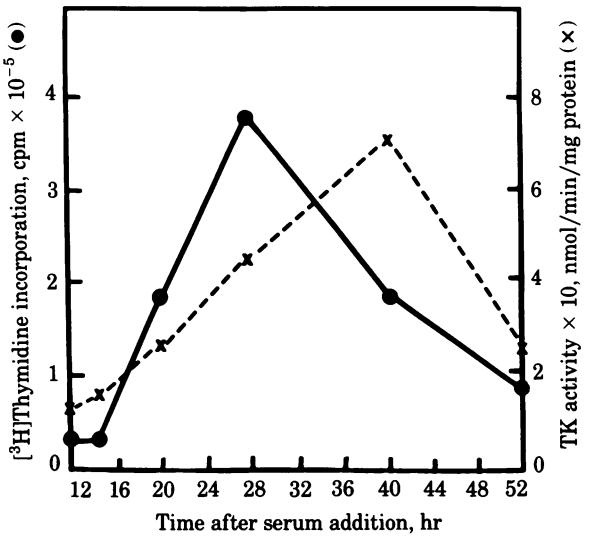

FIG. 5. Cell cycle-specific regulation of TK activity in LTK⁻ cells transformed to TK⁺ with λTK46 DNA. Cells were arrested in the G_1 phase of the cell cycle by serum deprivation. Upon addition of serum, the cells began to cycle as a synchronous population. DNA synthesis (●) was monitored by [³H]thymidine incorporation into acid-precipitable radioactivity, and TK activity (x) was measured.

small as 4.2 kb seems remote because, of 14 plaque-purified clones that hybridize to the 1.6-kb *Xho* I/*Eco*RI fragment, only λTK46 contains all the sequences required for TK⁺ transformation. This minimum-size estimate of the human TK gene exceeds the known size of the functional chicken TK gene (27) by a factor of 2.

In light of this finding, the 1.6-kb *Xho* I/*Eco*RI fragment was used as a hybridization probe to search for sequences homologous to the human TK gene in the DNA of other organisms. All primate DNAs examined (human, rhesus macaque, squirrel monkey, owl monkey, and lemur) contained strongly hybridizing bands after cleavage with *Sac* I. The length of the hybridizing fragments varied among primates, showing that the external *Sac* I sites are polymorphic. A *Sac* I digest of LTK⁻ DNA revealed a faintly hybridizing band of 5.2 kb only when the stringency of hybridization was reduced by 10°C (unpublished data).

Some peculiarities of the human TK gene have emerged from this study that explain the difficulties encountered by myself and others in cloning this gene. The functional TK gene is unexpectedly large when compared with viral and chicken genes. This complicates the task of recovering the entire gene on a single phage λ clone with a capacity of 20–24 kb. λTK46 was recovered as a partial digestion product of *Mbo* I, which has a tetranucleotide recognition sequence occurring every 256 nucleotides (on the average). At least 13 other clones contained a portion of the TK gene but could not be recognized by the biological assay of TK⁺ transformation. An alternate cloning strategy, avoiding the problem of overlapping but truncated clones, could have been tried. This method involves the preliminary identification of a restriction enzyme that does not abolish the TK⁺-transforming activity of human DNA. Based on the restriction endonuclease cleavage map of λTK46, the human TK gene is devoid of sites for *Bam*HI, *Bgl* II, or *Sal* I. Cloning of human DNA fragments generated by any of these enzymes would guarantee that any recombinant carrying TK gene sequences would be functional in transforming LTK⁻ cells to TK⁺. However, although the functional human TK gene lies completely within a single *Bam*HI, *Bgl* II, or *Sal* I fragment, these fragments may be too large to clone in phage λ vectors. The method used in this study is, therefore, more tedious but more general in its applicability. Even if the gene had not been recovered in functional form, sequences flanking the 6.4-kb *Sac* I fragment common to all primary and secondary transformants examined (Fig. 1) could have been subcloned and used as hybridization probes to clone the TK gene from an expressible cDNA library, as has been done for the human hypoxanthine phosphoribosyltransferase gene (28).

The most significant finding of this study is that the cloned 16-kb segment of human DNA containing the TK gene carries not only the TK sequences coding for protein but also harbors the control signals specifying the increase in TK activity observed at the onset of DNA synthesis. LTK⁻ cells transformed to TK⁺ with the cloned human TK gene show the S-phase induction of TK activity characteristic of normal TK⁺ cells.

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1. Bello, L. J. (1974) *Exp. Cell Res.* **89**, 263–274.
2. Lewin, B. (1980) in *Gene Expression* (Wiley, New York), Vol. 2, pp. 189–212.
3. Claycomb, W. C. (1979) *Exp. Cell Res.* **118**, 111–114.
4. McKnight, S. L. (1982) *Cell* **31**, 355–365.
5. Shih, C. & Weinberg, R. A. (1982) *Cell* **29**, 161–169.
6. Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. (1978) *Cell* **14**, 725–731.
7. Puck, T. T., Marcus, P. I. & Cieciura, S. J. (1956) *J. Exp. Med.* **103**, 273–284.
8. Mullins, J. I., Casey, J. W., Nicolson, M. O. & Davidson, N. (1980) *Nucleic Acids Res.* **8**, 3287–3305.
9. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
10. Wahl, G. M., Padgett, R. A. & Stark, G. R. (1979) *J. Biol. Chem.* **254**, 8679–8689.
11. Rozek, C. E. & Davidson, N. (1983) *Cell* **32**, 23–34.
12. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. J. (1977) *J. Mol. Biol.* **113**, 237–251.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
14. Sternberg, N., Tiemeier, D. & Enquist, L. (1977) *Gene* **1**, 255–280.
15. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
16. Vieira, J. & Messing, J. (1982) *Gene* **19**, 269–276.
17. Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1973) *Proc. Natl. Acad. Sci. USA* **69**, 2110–2114.
18. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
19. Dulbecco, R. & Vogt, P. (1954) *J. Exp. Med.* **99**, 167–182.
20. Lee, L.-S. & Cheng, Y.-C. (1976) *J. Biol. Chem.* **251**, 2600–2604.
21. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
22. Jelinek, W., Toomey, T., Leinwand, L., Duncan, C. H., Biro, P. A., Choudary, P. V., Weissman, S., Rubin, C., Houck, C., Deininger, P. L. & Schmid, C. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1398–1402.
23. Lowy, I., Pellicer, A., Jackson, J. F., Sim, G.-K., Silverstein, S. & Axel, R. (1980) *Cell* **22**, 817–823.
24. Lin, P.-F., Yamaizumi, M., Murphy, P. D., Egg, A. & Ruddle, F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4290–4294.
25. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280–289.
26. Schlosser, C. A., Steglich, C., deWet, J. R. & Scheffler, I. E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1119–1123.
27. Perucho, M., Hanahan, D., Lipsich, L. & Wigler, M. (1980) *Nature (London)* **285**, 207–210.
28. Jolly, D. J., Okayama, H., Berg, P., Esty, A. C., Filpula, D., Bohlen, P., Johnson, G. G., Shively, J.-E., Hunkapillar, T. & Friedmann, T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 477–481.