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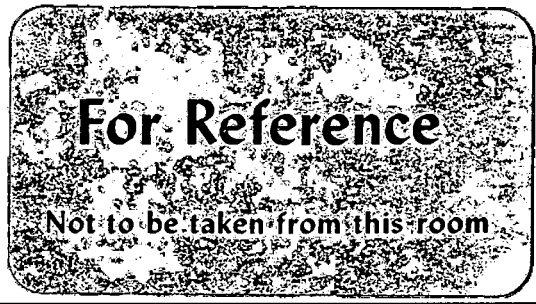
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IONIZING AND ULTRAVIOLET RADIATION ENHANCES
THE EFFICIENCY OF DNA MEDIATED GENE
TRANSFER in vitro

C.F. Perez
(Ph.D. Thesis)

August 1984



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IONIZING AND ULTRAVIOLET RADIATION ENHANCES
THE EFFICIENCY OF DNA MEDIATED
GENE TRANSFER in vitro

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Ph.D. Thesis

Lawrence Berkeley Laboratory
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August 1984

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IONIZING AND ULTRAVIOLET RADIATION ENHANCES THE
EFFICIENCY OF DNA MEDIATED GENE TRANSFER IN VITRO

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Carl F. Perez

Lawrence Berkeley Laboratory,
University of California

Ionizing and Ultraviolet Radiation Enhances the
Efficiency of DNA Mediated Gene Transfer in vitro

ABSTRACT

The enhancement effects of ionizing and non-ionizing radiation on the efficiency of DNA mediated gene transfer were studied. The established cell line, Rat-2 (Topp, 1981), consists of cells that are density dependent contact inhibited and produce flat monolayers in vitro. If these cells are infected with SV40 virus a small fraction of cells become morphologically "transformed". These transformants are not contact inhibited and will form colonies of piling-up cells, called foci, upon a monolayer of their "non-transformed" counterparts. This morphological phenotype is due to the expression of the SV40 virus A-gene. Rat-2 cells are competent for DNA mediated gene transfer (via calcium phosphate precipitation), deficient in thymidine kinase activity (TK⁻), and will die in HAT selective media.

Confluent Rat-2 cells were transfected with purified SV40 viral DNA, irradiated with either X-rays or ultraviolet, trypsinized, plated, and assayed for the formation of foci on Rat-2 monolayers. Both ionizing and ultraviolet radiation enhanced the frequency of A-gene transformants/survivor compared to unirradiated transfected cells. These enhancements were non-linear and dose dependent.

A recombinant plasmid, pOT-TK5, was constructed that contained the SV40 virus A-gene and the Herpes Simplex virus (HSV) thymidine kinase

(TK) gene. Confluent Rat-2 cells transfected with pOT-TK5 DNA and then immediately irradiated with either X-rays or 330 MeV/amu argon particles at the Berkeley Bevalac showed a higher frequency of HAT⁺ colonies/survivor than unirradiated transfected cells. In both cases the enhancement contained a linear and a higher order component in dose, but the argon ions were at least twice more efficient than X-rays in producing enhancement per unit dose. Rat-2 cells transfected with pOT-TK5, X-irradiated, and assayed for either TK-transformation or A-gene transformation showed the same dose dependence for radiation enhancement.

Rat-2 cells transfected with the plasmid, pTK2, containing only the HSV TK-gene were enhanced for TK-transformation by both X-rays and ultraviolet radiation. SV40 A-gene products are not necessary for the radiation enhancement of the efficiency of gene transfer.

Genomic analysis via Southern blotting and DNA-DNA hybridization was performed on extracted cellular DNA from 30 clones isolated from Rat-2 cultures transfected with pTK2 DNA. There was no significant enhancement in the amount of integrated plasmid DNA in clones arising from cultures that were X-ray irradiated post-transfection than from the unirradiated transfected cultures. Southern analysis of isolated nuclei from transfected Rat-2 cells, with and without X-ray treatment, showed no difference in plasmid DNA uptake. This result demonstrates that radiation enhancement of the efficiency of DNA mediated gene transfer is not explained by increased nuclear uptake of the

transfected DNA. Radiation does not increase the amount of DNA integrated per recipient individual cell, but it does increase the competence of the transfected cell population for genetic transformation.

Three models for this increased competence are presented. The targeted integration model states that radiation increases the production of DNA strand breaks, which are the presumed substrates for DNA integration. The inducible recombination model argues that radiation serves to induce the expression of DNA recombination genes that in turn increase the frequency of DNA integration. The partition model suggests that radiation does not increase the probability of DNA integration per cell, but changes the ability of the cell to express the integrated DNA. These three models and the utilization of DNA mediated gene transfer for DNA repair studies will be discussed.

Conclus J. T. Plein

Aug 1, 1984

DEDICATION

This work is dedicated to my parents Dr. and Mrs. F. M. Perez,
who have taught me the true value of love and the pursuit of truth.

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I would like to use this opportunity to acknowledge the financial support given me during my graduate career. I was supported by the University of California Regents Fellowship, NIH Radiation Biophysics Training Grant (Grant No. 5T32-CA90272), the Associated Western Universities Graduate Fellowship, the Cancer Research Coordinating Committee (Grant No. 1-523847-37849), and by a gift to the Donner Laboratory from the Cancer and Medical Research Foundation, Inc. of San Francisco. My research was also supported in part by the Department of Energy (DOE Contract No. DE-AC03-76SF00098) and the National Cancer Institute (Grant No. CA15184).

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INTRODUCTION

The objective of my research was to investigate the molecular mechanism involved in the ionizing radiation enhancement of SV40 transformation of rodent cells in culture. The following discussion traces the motivation in 1) studying radiation induced transformation in vitro, 2) using SV40 tumor virus in radiation co-carcinogenic studies, and 3) proposing the construction of a recombinant SV40 virus.

HISTORICAL PERSPECTIVE

The first record of a radiation induced tumor in a human being dates from 1902 by Frieben, seven years after the discovery of X-rays by Roentgen. Frieben described a neoplasm in an X-ray induced ulcer on the hand of Clarence Madison, a radiation worker in Thomas Edison's Laboratory (Brown, 1936). In 1911, Von Jagie reported the incidence of five leukemias in radiation technicians. Since these first reports, more data has been amassed concerning radiation induced human neoplasia. Radium dial painters who ingested radium salt had higher incidences of osteosarcomas than the normal population (Martland, 1931; Looney, 1958). Epidemiological data has been incontrovertible for radiation as the cause of certain neoplasms: 1) increased leukemia from patients irradiated to treat ankylosing spondylitis (Court Brown and Doll, 1965), 2) increased breast cancer following multiple fluoroscopies (Mackenzie, 1965; Myrden and Hiltz, 1969), 3) increased thyroid cancer in natives exposed to radioactive iodines in fallout from hydrogen bomb test explosions (Conard et al., 1970), and 4) increased incidence of cancer among the survivors of the Hiroshima and

Nagasaki atomic detonations (Ishimaru et al., 1966; National Academy of Sciences, 1972; BEIR, 1972; and UNSCEAR, 1977, 1984).

Most experimental studies in radiation carcinogenesis have been animal studies (Fry and Ainsworth, 1977; Ullrich et al., 1977; Upton, 1964, 1975; Bond et al., 1960; Kaplan, 1967; Storer, 1975, Brues, 1951). The data from these studies have been predominantly qualitative due to the statistical limitations imposed by performing experiments with relatively small numbers of animals. The molecular and cellular effects of radiation cannot be investigated macroscopically no matter how many animals are tested. These shortcomings led to the establishment of cell culture systems. The aims of this approach were to standardize conditions of environment and trauma and to create the milieu necessary to study gene expression, cell-cell interactions, and ultimately the neoplastic process itself.

Alexis Carrel in 1925 suggested that "the best method of ascertaining properties that characterize a malignant tissue would be to transform in vitro a strain of cells of a known type into cells capable of producing sarcoma or carcinoma and to study the changes undergone by the strain." Puck and Marcus developed a clonal assay that served as the foundation mammalian cell of radiation biology (Puck and Marcus, 1956; Puck et al., 1956; Puck, 1958). They demonstrated a dose-related effect of X-ray irradiation on the survival of single cells. The frequency of radiation induced transformation would then be deduced from these values. Borek and Sachs (1966) first reported the direct oncogenic effects of X-rays by exposing diploid

hamster embryo cells to 300 rads (3.00 Grays) of X-rays and transforming a fraction of these cells into cells which were morphologically different from their unchanged counterparts. These transformed cells produced tumors when injected into hamsters, while the control cells did not. Radiation induced transformation of mammalian cells in vitro was extended to mouse cell systems (Terzaghi and Little, 1976; Little, 1979; Han and Elkind, 1979; Miller and Hall, 1978) and human cells (Borek, 1980).

The primary events that lead to neoplasia are difficult to detect in cells, and even harder in tissues. We are relegated to studying the phenotypes of oncogenic expression. Oncogenic "transformation" appears similar regardless of the transforming agent: chemicals, ionizing and nonionizing radiation, and viral insertions of genetic material. Various criteria distinguishing "normal" cells from "transformed" cells have been studied over the years.

CRITERIA FOR TRANSFORMATION.

I have collated many of the commonly used criteria for transformation in TABLE I. Compared to normal cells, transformed cells show: 1) greater cloning efficiency, 2) unlimited mitotic potential, 3) higher saturation density in culture and the ability to "pile-up" upon one another, 4) growth in liquid and agar suspensions, 5) lower growth requirements, 6) aneuploidy and chromosome rearrangements, 7) membrane and cytoskeletal changes, 8) increases in proteolytic enzyme levels, and 9) the ability to form tumors when injected into a suitable

Table I
 Characteristics of Normal Cells and Tumor Cells in vitro

CRITERION	NORMAL CELLS	TUMOR CELLS	REFERENCES
1. Cloning efficiency	low	high	Puck (1958)
2. Culture Behavior	contact inhibited with low saturation density and growth in monolayer	high saturation density and showing piling-up	Abercrombie Heaysman (1954) Stoker Rubin (1967) Temin Rubin (1958) Aaronson Todaro (1968) Abercrombie (1966) Todaro et al. (1964) Berwald Sachs (1963) Borek Sachs (1966) Reznikoff et al. (1973)
3. Mitotic Potential	limited	unlimited	Hayflick Moorehead (1961) Ferguson Wangbrough (1962) Dulbecco Vogt (1960)
4. Glycolysis	low	high	Warburg (1956) Broadfoot et al. (1964)
5. Growth in liquid and agar suspension	no	yes	MacPherson Montagnier (1964) Borek Sachs (1966) Borek and Hall (1973, 1974) Lloyd et al. (1979) Borek (1980) Kakunaga (1978) Sutherland et al. (1980) Silinskas et al. (1981)

Table I (continued)

CRITERION	NORMAL CELLS	TUMOR CELLS	REFERENCES
6. Serum requirement for growth	high	low	Temin (1967) Jainchill Todaro (1970) Borek et al. (1977) Terzaghi et al. (1976) Dulbecco (1970)
7. Karyotype	Diploid	Aneuploid, rearrangements instability	Beatty (1957) DiPaolo Donovan (1967) Bloch-Schacter Sachs (1976) Kinsella Radman (1978) Schimke et al. (1981) DiPaolo et al. (1971)
8. Calcium requirement for growth	high	low	Swierenga et al. (1978)
9. Agglutination by low concentration of some plant lectins	low	high	Burger Martin (1972) Inbar Sachs (1969) Borek et al. (1973) Shoham Sachs (1974) Mannino Burger (1975) Nicholson (1974)
10. <u>Proteolytic enzyme increase</u>			
Plasminogen activator	no	yes	Borek et al. (1978) Unkeless et al. (1973)
acid phosphatase	no	yes	Jones et al. (1975) Borke et al. (1977)
serine protease MIF factor	no	yes	Borek et al. (1979) Poste (1975)

Table I (continued)

CRITERION	NORMAL CELLS	TUMOR CELLS	REFERENCES
11. Membrane Structural Changes			
Neoantigen	no	yes	Embelton Heidelberg (1975) Klein Klein (1967)
Glycoproteins	high	low	Gahnberg Hakomori (1973)
Gangliosides	high	low	Brady et al. (1969) Brady Fishman (1976)
Na ⁺ /K ⁺ ATPase	low	high	Borek Guernsey (1981)
LETS protein	present	none	Hynes Destree (1978) Gahnberg Hakomori (1973)
Cytoskeleton	organized	disorganized	Porter (1975)
12. Tumorigenicity	No	Yes	Sanford (1958) Klein Klein (1967) Kakunaga (1978) Milo DiPaolo (1978) Borek (1980) Sutherland et al. (1980) Silinskas et al. (1981) Borek et al. (1977)

animal host. Unfortunately, every transformed clone does not exhibit every one of these criteria, nor to the same degree. This fact is not surprising if one is embracing the multi-step model of oncogenesis. According to this model, cells after initiation events progress through different stages of transformation until a malignant phenotype is attained. The criteria we score as "transformation" may only be secondary events in the true progression of oncogenesis. With these caveats in mind, I will discuss the systems used to study in vitro transformation.

CELL CULTURE SYSTEMS.

There are three culture systems presently being used in radiation transformation studies: 1) primary cultures, 2) established cell lines, and 3) in utero-in vitro cultures.

Primary culture cells are direct descendants of animal or human tissue cells. The cells most commonly used are from hamster embryos (Borek and Sachs, 1966; Borek and Hall, 1973; DiPaolo et al., 1976). Human primary cultures that are often used for transformation studies are fibroblasts derived from human skin (Kakunaga, 1978; Borek, 1980), human foreskin (Milo and DiPaolo, 1978; Silinskas et al., 1981), and human embryos (Sutherland et al., 1980). The advantages of primary cultures are that they are diploid and senesce upon serial sub-culturing. Transformed clones are identified as dense multi-layered cells in random cellular arrangement and haphazard cell-cell orientation, whereas "normal" clones are flat with an organized cell-to-cell organization. Due to the rapid senescence and distinct morphological

criterion for transformation, survival and transformation are scored simultaneously on the same culture dishes. The spontaneous transformation frequency in these cells is less than 10^{-6} .

The obvious disadvantage with this system is the need to produce fresh primary cultures for every experiment, though these cells can be frozen in liquid nitrogen (Pienta et al., 1977). Another disadvantage is the dissolution of embryos that produces a mixed population of cell types that may possess a higher cell density than the usual flat colonies. These denser colonies would not demonstrate the random orientation at the colony edge which is possessed by transformed colonies.

Established cell lines have unlimited life spans. These cells originated from primary cell cultures that have been serially subcultured many times. The subcultured cells enter a "crisis" phase of decreasing plating efficiency from which a subpopulation of cells emerge that are "immortal." The two most common cell lines used for in vitro transformation studies are the Balb/C 3T3 cell line established by Todaro and Green (1963) and the 10T1/2 cell line established by Reznikoff and collaborators (1973) in Heidelberger's laboratory. Both of these cell lines were established from mouse embryo cells, are transformable by a variety of oncogenic agents, and are used extensively for radiation studies. Unlike the hamster embryo primary cell system, the maintenance of cell lines does not require an animal breeding colony. Cell lines can be cryopreserved so that experiments may be performed on the same low passage number. One of

the disadvantages of the fibroblast cell line system is that if cells are not subcultured as carefully as originally described, they give rise to spontaneous transformants at high passage numbers. These established cell lines are not diploid, and their karyotype is characterized by various chromosomal rearrangements and heteroploidy. Transformation is scored by the appearance of foci of piling-up cells on a monolayer of density-dependent contact inhibited normal cells. Radiation induced transformation studies require two sets of plates: one set for plating efficiency and surviving fraction, and the other set for transformed foci. The foci are morphologically scored as types I, II, and III (Reznikoff et al., 1973; Terzaghi and Little, 1976), with type III being the most malignant.

The third system, and one not extensively used, is the in utero in vitro system devised by Borek and coworkers (1977). Hamster embryos are irradiated in utero and scored for transformation events in vitro.

Before proceeding to the experimental results of radiation induced transformation studies, we must discuss two aspects of radiation biology: radiation quality and radiation damage.

Radiation Quality. Ionizing radiation can be distinguished by its ability to deposit energy to the media in which it travels along the length of its track. This concept of linear energy transfer (LET) was suggested by Zirkle in 1940 and defined by Zirkle and Tobias in 1953. LET is measured in units of KeV/ μm . With early detection techniques, LET was measured in two ways: 1) track averaged, in which the energy

along an increment of track was measured, 2) energy average, in which the length of track was measured for a particular increment of energy. The LET's measured by these two techniques might vary, especially when the critical "cut-off" energy value was restricted to different values (International Commission on Radiation Units, 1970). Presently calculation and measurement of linear energy transfer have attained great sophistication. In the present radiation biophysics literature we refer to an LET_{∞} , which is the linear energy transfer given completely to the media in radial direction perpendicular to the track. In general, sparsely ionizing radiation such as gamma rays and X-rays have LET_{∞} 's (track averaged) of 0.2 to 7 KeV/ μ m.

Heavy charged particles from accelerators may have LET_{∞} 's of 43 KeV/ μ m (5.6 MeV/amu helium ions), 200 KeV/ μ m (600 MeV/amu iron ions), to even 2000 KeV/ μ m (8.0 MeV/amu argon ions).

Radiation Damage. In the available radiobiological literature, radiation induced damage has been operationally classified as based on expressed endpoints into three main categories: 1) lethal damage, 2) sublethal damage, and 3) potentially lethal damage.

A standard radiobiological approach is found in the split-dose experiment. Introduced by Elkind and his collaborators (Elkind and Sutton, 1959; 1960; Elkind and Sinclair, 1965; Elkind, 1967; Elkind, et al., 1965), these experiments proceed by the irradiation of a cell population with a first dose, then administering a second dose after various periods of time. The concept of sublethal damage emerged after the results were studied that cells irradiated with a first dose

and allowed to recover before the second dose demonstrated a higher survival than if both doses were administered at one time. A population of cells exposed to a small dose of radiation may fall into three categories:

- 1) The ionizing event may not have occurred in any of the critical inactivation targets, leaving the cell undamaged.
- 2) The ionizing event may have occurred in all critical inactivation target sites, producing lethal damage, and rendering the cell unable to maintain reproductive integrity.
- 3) A cell may have sustained ionizing events in some, but not all, of its critical target sites. This cell is said to have sustained sublethal damage. It has been damaged but it has not been "killed." Given time, the cell may be able to repair the effects of this sublethal damage and completely recover from it. The time period required for the repair of sublethal damage is approximately 1 hour.

Potentially lethal damage is a measure of how environmental conditions experienced by the cell post-irradiation can affect cell survival. This effect was first studied in HeLA cells by Phillips and Tolmach (1966). Operationally, the radiation response was observed when biological conditions of growth (37°C, full growth media, standard pH, no over-crowding, etc.) are described as "normal." The fraction of cells that survive a particular dose may be above or below the normal response, depending upon whether the potentially lethal damage is repaired or expressed, respectively. Cells that are

irradiated in monolayer and trypsinized and plated after a few hours post-irradiation show increased survival--PLD has been repaired. Cells that have been treated with certain DNA synthesis inhibitors post-irradiation show decreased survival--more PLD has been expressed.

To summarize, lethal damage is irreversible, irreparable damage that leads to cell death. Sublethal damage can be repaired under normal conditions in a few hours, unless additional sublethal damage is induced, which may interact to produce lethal damage. Potentially lethal damage is that part of radiation damage that can be influenced by post-irradiation conditions (e.g., delayed plating, treatment with drugs). These distinctions are operational terms, since the exact nature and interaction among these damages are unknown.

EXPERIMENTAL RESULTS OF RADIATION TRANSFORMATION IN VITRO.

What have we learned from these in vitro studies about radiation induced transformation? Sparsely and densely ionizing radiations induce transformation. Using the hamster embryo primary cell system, Borek and Sachs (1966) showed that X-rays produce transformants. Later Borek and collaborators (1978) showed that 430-KeV monoenergetic neutrons and argon ions (produced from the BEVELAC) induced transformation. Borek (1977) reported that X-rays did induce transformation in hamster embryo cells irradiated in utero, but that the transformation frequency was lower than the frequency observed if the cells were irradiated in vitro. The 10T1/2 cell line system has been extensively used for chemical carcinogen studies (Reznikoff et al., 1973) and TABLE II cites many of the

studies conducted with various radiation modalities: ultraviolet, X-rays, fission neutrons, and heavy charged particles. In general, radiation induces transformation in 10T1/2 cells.

Split dose experiments studying sublethal damage repair effects on transformation frequency produced interesting results in both the hamster embryo and 10T1/2 systems. Borek and Hall (1974) showed in split dose experiments with the hamster embryo system that the transformation frequency increases at doses less than 1.0 Gray per survivor but decreases at high doses (Borek, 1979b). Similar results were reported for the 10T1/2 cell line system. Miller and Hall (1978) reported that the transformation frequency increased when the split dose experiments were performed with low doses, while Han and Elkind (1979a) reported decreased transformation frequency per survivor at split doses in the high dose range.

Low dose experiments have been performed with the 10T1/2 cell line system with conflicting results. Although lower transformation frequencies have been reported by Han and collaborators (Han and Elkind, 1979a; Han et al., 1980), Hall and Miller (1981) reported higher transformation frequencies at low dose rates versus high dose rates. The difference in results may be attributed to the experimental conditions. Han and coworkers irradiate the 10T1/2 cells in vitro, then trypsinize and plate, while Hall and Miller trypsinize and plate the cells and then irradiate the single cells in vitro.

Table II
In Vitro Transformation of C3H10T1/2 Cells

Radiation	LET (KeV/u)	DOSE Grays	Number of transformants per survivor (x 10 ⁴)	References
UV	---	25-300 erg/mm ²	1 - 10	Chan Little (1976)
X-Rays (50 Kvp)	6.3*	.5-12	.8 - 30	Han Elkind (1979)
X-rays (100 kVp)	4*	.5 - 12	.3 - 30	Terzaghi Little (1976)
X-rays (225 kVp)	2*	1 - 8	2 - 100	Yang Tobias (1980)
Fission Neutrons (0.85 Mev)	---	.25 - .60	1 - 60	Han Elkind (1979)
Carbon Ions (470 MeV/n)	10-34	.5 - 10	50 -200	Yang et al. (1981)
Helium Ions (5.6 Mev)	43	2 - 3.4	400	Lloyd et al. (1979)
Silicon Ions (240 MeV/n)	84	.5 - 5	3 - 36	Yang et al. (1983)
Carbon Ions (13.2 MeV/n)	128	.5 - 3	3 - 28	Yang et al. (1982)
Iron Ions (600 MeV/n)	190	.1 - .9	5 - 60	Yang Tobias (1980)

* Track-averaged LET

Despite the convenience of the 10T1/2 system, experiments may take as long as five to six weeks to complete. Large numbers of vials containing low passage numbers of cells must be stored in liquid nitrogen. The number of transformed clones scored is small compared to the number of dishes that are incubated and fed with media. Consequently, the small number of transformants makes significant statistical inferences, at best, extremely difficult.

RADIATION STUDIES WITH SV40 TUMOR VIRUS.

The approach pursued by Yang and his collaborators involved studying the effect of radiation on cells that were infected with simian virus 40 (SV40). SV40 was discovered in human polio vaccines produced in rhesus monkeys. The virus was not originally detected because it did not produce any cytopathic effects in rhesus monkeys. But Sweet and Hilleman (1960) infected cells from a species of African green monkeys of the genus Cercopithecus and found immediate cytopathic effects. It was clear that SV40 was present in the vaccines that inoculated many millions of human beings. Soon after it was shown that SV40 could produce tumors in animals injected with the virus (Eddy et al., 1962; Girardi, et al., 1962). Fortunately, follow-up studies on the inoculated human population have not shown any increase in cancer frequencies compared to the control population.

The biological and physical characterization of SV40 proceeded rapidly. Black and collaborators (1964) were able to produce and purify substantial amounts of the virus. Todaro and Green (1964)

first demonstrated that SV40 could transform mouse 3T3 cells in vitro, followed by Black (1966) who established the relationship between the multiplicity of infection and the transformation response.

SV40 is a member of the papovavirus family, which also contains polyoma and papilloma viruses. The SV40 virus particle is 45 nm in diameter and has a molecular weight of 27 megadaltons. Each virion contains one covalently closed, double-stranded DNA circle which is associated with about 21 host nucleosomes. This DNA-protein complex is surrounded by an icosahedral capsid composed of three types of virally coded proteins (Finch and Klug, 1965; Anderer et al., 1967; Koch et al., 1967). The 5243 base-pair sequence (Reddy et al., 1978; Fiers et al., 1978) codes for at least five proteins: the tumor antigens large T and small t, and the three viral capsid proteins VP1, VP2, and VP3. A thorough review of SV40 biology may be found in Tooze (1980).

Pollack and Todaro (1968) showed that SV40 viral transformation of infected 3T3 mouse cells was significantly enhanced when the cells were infected either before or immediately after X-irradiation. Later the enhancement of viral transformation by ultraviolet light was reported by Lytle et al. (1970). At this time there was good experimental evidence that SV40 viral DNA was persisting stably in transformed cells. Co-culturing SV40 transformed cell lines with uninfected permissive cell lines (such as CV-1, AGMK) produced infectious viral particles (Gerber and Kirchstein, 1962). Gillespie and Spiegelman

(1965) used radioactive virus-specific RNA to hybridize to transformed cellular DNA and they found that more hybridization occurred among the transformed cellular DNA than the untransformed cellular DNA. By reconstruction experiments, Westphal and Dulbecco (1968) concluded that multiple genome equivalents of SV40 existed in many transformed cell lines. Gelb and collaborators (1971) used DNA-DNA hybridization techniques (the highly quantitative Cot analysis) to determine that transformed cell lines contain as little as one to three copies of SV40 DNA. Transformed cellular DNA was cut with restriction endonucleases (see Appendix C), electrophoresed in agarose gels, transferred to nitrocellulose filters, and hybridized with radioactive probes by the techniques developed by Southern (1975). Ketner and Kelley (1976) examined several SV40 transformed cell lines and reported different restriction patterns of DNA fragments that hybridized to SV40. Botchan and coworkers (1976) studied several SV40 transformed cell lines and showed that SV40 DNA did not have a specific integration attachment site, nor did the SV40 DNA integrate at any specific site in the cellular genome.

Yang and collaborators (1980, 1981, 1983) investigated the kinetics of SV40 viral transformation enhancement with sparsely and densely ionizing radiation. TABLE III summarizes the various radiation modalities that enhance SV40 transformation of rodent cells in vitro. These transformation experiments score foci on contact-inhibited monolayers. The transformed clones that arise in fourteen days are attributable to SV40 transformed cells and not to radiation transformed

Table III
Enhancement of SV40 Transformation

Radiation	LET (KeV/u)	DOSE	Enhancement Ratio	Cells	References
UV	---	150-440 ergs/mm ²	1 - 6	3T3	Lytle et al. (1970)
UV	---	75 ergs/ mm ²	3.7	C3H2K	Ide et al. (1975)
Caffeine	---	1 mM	6.0	C3H2K	"
UV + Caffeine	---	"	16.7	C3H2K	"
X-rays (250 kVp)	2*	3-12 grays	1 - 7	3T3	Pollock Todaro (1968)
X-rays (225 kVp)	2*	1-6 Grays	1 - 16	10T1/2	Yang et al. (1980)
Carbon Ions (400 Mev/n)	11	1-6 Grays	1 - 6	10T1/2	Yang et al. (1981)
Neon Ions (425 Mev/n)	34	.5-4 Grays	1 - 20	10T1/2	Yang et al. (1981)
Argon Ions (570 Mev/n)	80-90	.5-3.5 Grays	1 - 5	10T1/2	Yang et al. (1981)
Carbon Ions (7.5 Mev/n)	200	1.4 grays	25	10T1/2	Yang et al. (1981)
Argon Ions	2000	1.3 grays	0.5	10T1/2	Yang et al. (1981)

* Track averaged LET

foci, which normally cannot be scored for another 21-28 days. The transformation frequency per survivor increases as the square of the X-ray dose, suggesting two-hit kinetics. Heavy ions with LET's greater than 20 KeV/ μ m are more efficient than X-rays in producing enhancement. Heavy ions with LET's above 100 KeV/ μ m increase the frequency of transformants per survivor as a linear function of dose. At extremely high LET's (greater than 2000 KeV/ μ m), the enhancement is actually attenuated. This effect is most likely attributed to the very large inactivation cross-section of the densely ionizing particle. Most particles hitting the nucleus cause a lethal event, but some cells survive that can undergo enhancement.

Radiation repair experiments have been performed on the enhancement of SV40 transformation. Xeroderma pigmentosum cells which cannot repair ultraviolet damage (Cleaver, 1973) cannot be enhanced for SV40 transformation by ultraviolet (Key and Todaro, 1974). Yang et al. (1980) performed split-dose experiments to ascertain the effect of the repair of sublethal damage. 10T1/2 cells were infected with SV40 virus and then given two fractions of X-rays eight hours apart. Survival increased with the repair of sublethal damage, but the enhancement of SV40 transformation decreased. Yang et al. also performed potentially lethal damage experiments in the same study. 10T1/2 cells infected, irradiated, and plated 18 hours post-irradiation showed the same enhancement as unirradiated infected controls. They concluded that given enough time, the lesions involved in the enhancement of viral transformation can be repaired. Ide and coworkers (1975)

demonstrated that combined ultraviolet and caffeine treatments of SV40 infected 10T1/2 cells enhanced transformation to a greater extent than any one of these treatments separately. Cleaver and Thomas (1969) suggested that caffeine interferes with postreplication repair.

To summarize the data to 1980, we see that:

1. SV40 transformed cells contain integrated viral DNA.
2. X-rays and ultraviolet enhance SV40 transformation as a quadratic function of dose.
3. Heavy ions enhance SV40 transformation even more. At LET's greater than 100 KeV/ μm , the enhancement is a linear function of dose.
4. Potentially lethal damage repair decreases enhancement.
5. Sublethal damage repair decrease enhancement.

The simplest working hypothesis formulated by the researchers ran as follows:

ENHANCEMENT IS DUE TO INCREASED SV40 DNA INTEGRATION AT SITES OF RADIATION INDUCED LESIONS IN CELLULAR DNA.

What role does T-antigen play in this radiation enhancement?

Non-permissive cells infected with SV40 show increases in the specific activity of enzymes involved in DNA synthesis: thymidine kinase, DNA polymerase, dCMP deaminase, dihydrofolate reductase, and thymidylate synthetase (Kit et al., 1966ab, 1967ab; Frearson et al., 1966). Tjian and coworkers (1978) microinjected SV40 T-antigen into cells and induced cellular DNA synthesis, proving that T-antigen can alter DNA metabolism.

It is possible to isolate cells containing SV40 DNA and expressing T-antigen (as measured by immunofluorescence) that do not display the transformed phenotype. These cells were generated from SV40 transformed cells that were treated with cell killing agents and selected for reversion for density inhibition (Pollack et al., 1968; Pollack and Burger, 1969; Culp et al, 1971; Culp and Black, 1972; Risser and Pollack, 1974). While these revertants show lesser degrees of transformed phenotype (e.g., lower growth in methocel, less tumorigenic), they do contain SV40 DNA and infectious virus can be rescued from them. The implications are that T-antigen is necessary for transformation but it is not important to maintain transformation. Another theory suggests that despite its antigenic response to anti-sera, the t-antigen found in revertants is mutated and is unable to maintain the transformed phenotype. The rescue of infectious virions from revertants via cell-fusion experiments nullifies this argument. Another model suggests that a cellular gene necessary for transformation has been altered instead.

Under more stringent conditions for revertant production, Steinberg and coworkers (1978) demonstrated that their revertants isolated by 5-fluoro-2'deoxyuridine selection, completely lack T-antigen, and that in some of these revertants the single-copy viral genome has been deleted or altered.

Nevertheless, the fact remains that some cells may have integrated SV40 DNA and express T-antigen and not be transformed under the criteria used for in vitro studies. I became interested at that point

in constructing a recombinant SV40 virus that would contain a marker gene whose expression could be scored unambiguously (see Chapter one).

HAT SELECTION FOR THYMIDINE KINASE ACTIVITY

The HAT (Hypoxanthine, Aminopterin, Thymidine; Szybalski and Szybalska, 1962) selection system for the activity of thymidine kinase (TK; E.C. 2.7.1.21, ATP: thymidine 5'-phosphotransferase) is well characterized. The basis for the selection system is as follows: aminopterin (4-aminopterolyglutamic acid) inhibits dihydrofolate reductase (E.C. 1.5.1.3, 5,6,7,8-tetrahydrofolate: NADP⁺ oxidoreductase) (Hitchings and Burchall, 1965), which converts dihydrofolate to tetrahydrofolate (THF) (see Figure I.1). In the absence of the co-factor THF, methylene-tetrahydrofolate (methylene-THF) cannot be produced by the transfer of the carbon moiety of serine to THF catalyzed by serine transhydroxymethylase (E.C. 2.1.2.1, 5,10-methylene-tetrahydrofolate: glycine hydroxymethyl-transferase). Concomitantly, in the absence of methylene-THF, dUMP (2'-deoxyuridine-5'monophosphate) cannot be methylated to dTMP (thymidine-5'-monophosphate) by the action of thymidylate synthetase (E.C. 2.1.1.b. 5,10-methylene-tetrahydrofolate: dUMP C-methyltransferase) (Cohen et al., 1958; Mathews and Cohen, 1963). Cells are able to grow in aminopterin if supplied with hypoxanthine, glycine, and thymidine since they contain hypoxanthine-guanine phosphoribosyl transferase (HGPRT; E.C. 2.4.2.8, IMP: pyrophosphate phosphoribosyltransferase), which catalyzes the conversion of hypoxanthine into purines, and thymidine kinase, which

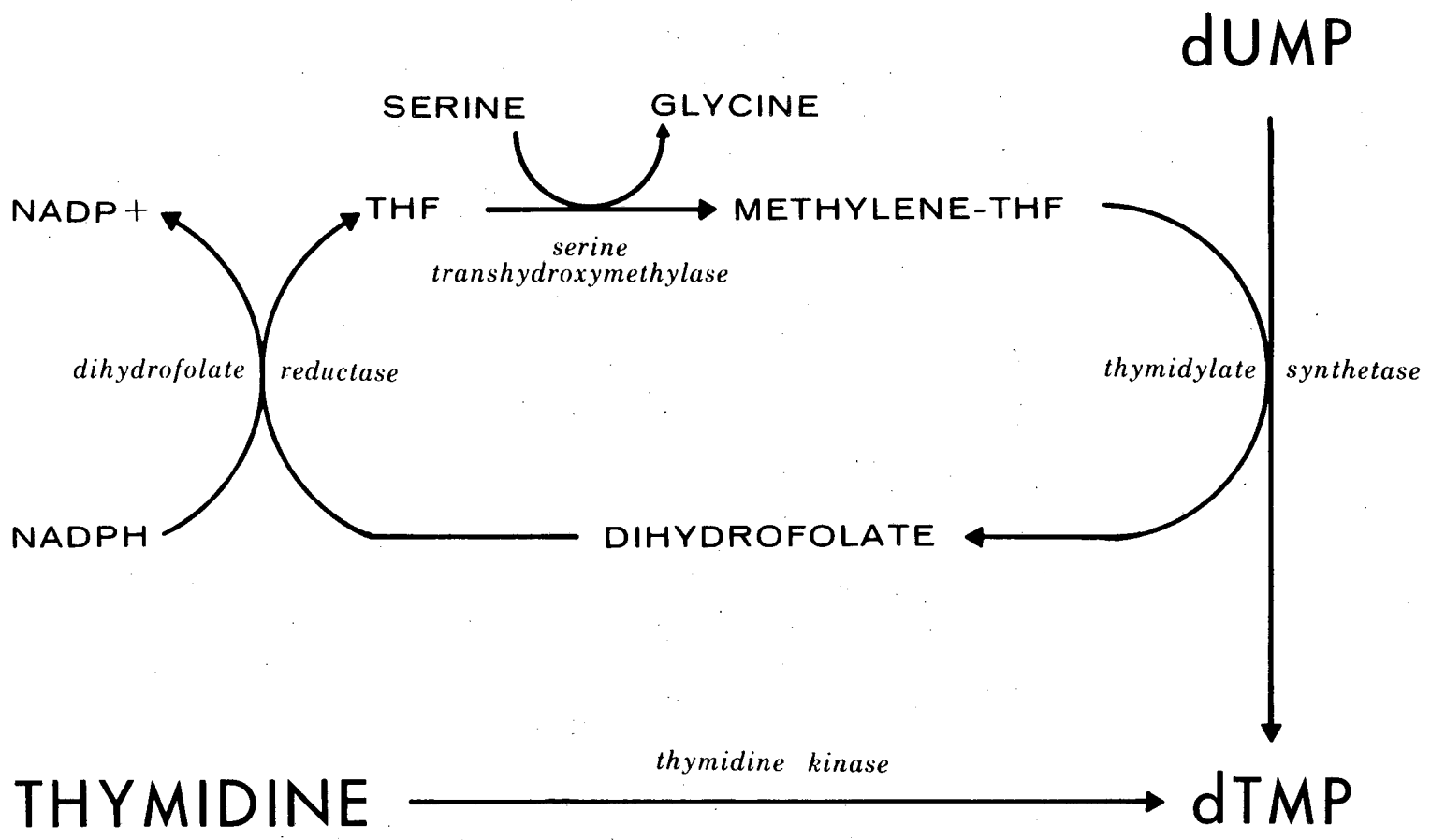


Figure I.1. Synthesis of thymidine-5' monophosphate (dTMP).

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converts thymidine to dTMP (1959) (Hakala and Taylor). The HGPRT and TK enzymes are not essential for cell growth under normal conditions since they are not involved in the major biosynthetic pathways for purine and dTMP (Hakala and Taylor, 1959). The selection media containing hypoxanthine, aminopterin, and thymidine (as well as glycine) was called HAT media by Szybalska and Szybalski (1962). Cells which lack HGPRT and/or TK activity will die in HAT media.

Cells deficient for HGPRT activity can be isolated from normal cell lines on the basis of their resistance to the growth inhibitory effect of purine analogs such as 8-azaguanine (Littlefield, 1963) or 6-thioguanine (Goldstein and Lin, 1972). On the other hand, cells deficient for TK activity can be isolated from cells selected for their resistance to 5-bromodeoxyuridine (Kit et al., 1963).

It has been well established that herpes simplex viruses code for a thymidine kinase (Klemperer et al., 1967; Kit, 1976). The gene that codes for thymidine kinase can be transferred to thymidine kinase-deficient cells by infection with UV-inactivated virus (Munyon et al., 1971) or with temperature sensitive mutant viruses (Hughes and Munyon, 1975); or by DNA mediated gene transfer of purified HSV viral DNA (Wigler et al., 1977; Minson et al., 1978; Bacchetti and Graham, 1977), purified HSV DNA fragments (Maitland and McDougall, 1977), purified restriction fragments from cloned HSV TK-gene inserted in bacterial plasmids (Enquist et al., 1979; Colbere-Garapin, et al., 1979), and a cellular DNA from cells transformed by HSV DNA (Minson

et al., 1978). The viral thymidine kinase enzyme has been purified (Cheng and Ostrander, 1976) and can be easily distinguished from cellular TK's on the basis of immunological (Klemperer et al., 1967), electrophoretic (Kit et al., 1974, 1975), and substrate-specific properties (Cooper, 1973; Summers and Summers, 1977; Smiley et al., 1980).

RESEARCH PROPOSAL: construct a SV40-TK recombinant virus.

Michael Botchan proposed (personal communication) the construction of a recombinant SV40 virus to study SV40 integration. The virus would contain the HSV thymidine kinase gene and a mutant SV40 A-gene (from mutant ptsA-209, Chou and Martin, 1974) that codes for a temperature sensitive T-antigen. The virus would be used to infect the rodent cell line Rat-2 (Topp, 1981), an ideal cell line for these studies. The Rat-2 cell line is: 1) flat and density-dependent contact inhibited, 2) transformable by SV40 A-gene via viral infection or DNA mediated gene transfer, 3) able to grow at 33°C and 37°C, the permissive and non-permissive temperatures for the tsA-209 mutant, and 4) thymidine kinase deficient.

Rat-2 cells will be infected with recombinant SV40-TK virus and then scored for thymidine kinase activity by HAT selection or SV40 A-gene activity by focus assay. With this new system these experiments are planned:

1. Investigate the predicted ionizing and ultraviolet radiation enhancement of transformation by both marker genes.

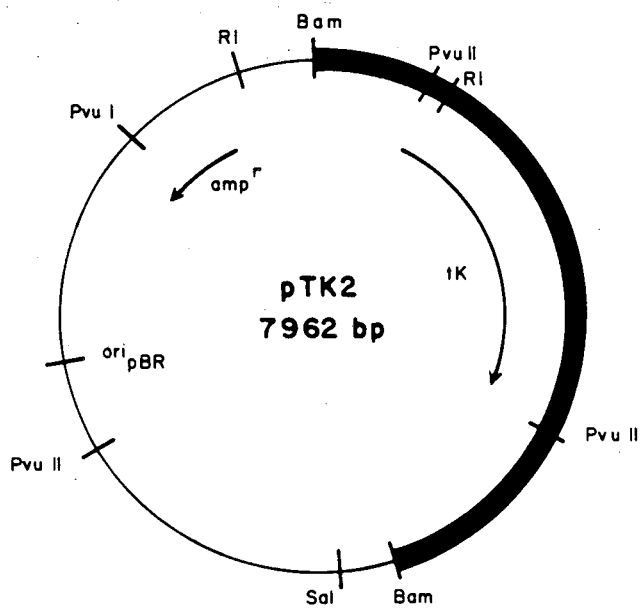
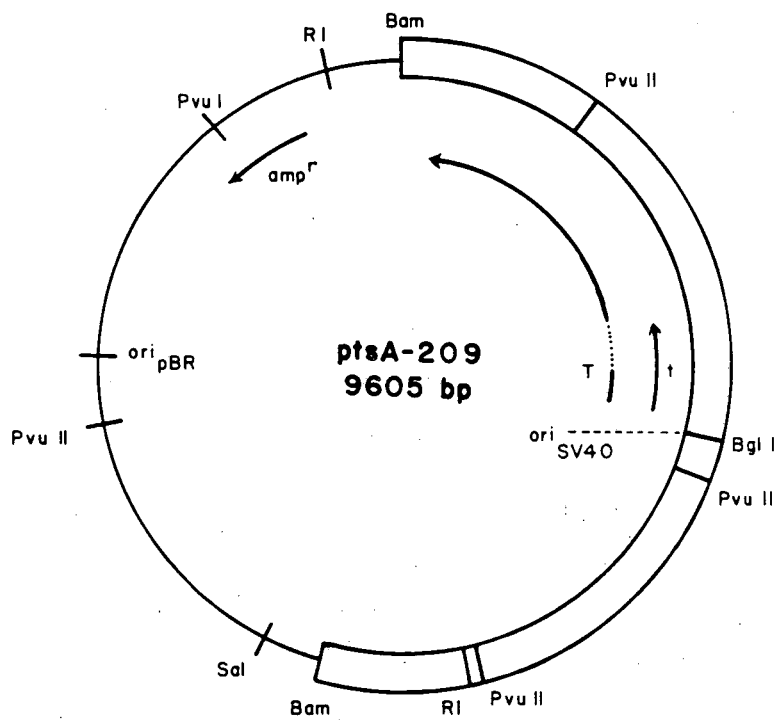
2. Perform temperature shift experiments to delineate the role, if any, of SV40 T-antigen in the enhancement of transformation.
3. Analyze quantitatively and qualitatively the integrated viral DNA in the cellular DNA isolated from transformants.

CHAPTER ONE

CONSTRUCTION OF A RECOMBINANT SV40-TK DNA PLASMID

The SV40-TK recombinant DNA used for my research was constructed by employing the techniques found in Appendices B,C,D, and E. These techniques involved inserting foreign DNA into specifically engineered bacterial plasmids that confer antibiotic resistance to competent bacteria. The bacterial clone containing the desired plasmid with the DNA insert is isolated by in situ DNA hybridization. The strategy I used was first to clone the tsA-209 viral DNA into a bacterial plasmid, pBR322, and then to insert the herpes simplex viral thymidine kinase gene into this plasmid (figure 1.1). The full account of the construction is presented in Appendix F.

A packaging restraint is imposed by the SV40 viral system that dictates the amount of DNA that can be efficiently encapsidated. When SV40 virus is serially passaged at high multiplicities of infection, defective virions are produced. The contour lengths of the defective viral DNA from these virions were studied by electron microscopy (Yoshike et al., 1975; Tai et al., 1972). The maximum length of DNA from defective virions was at most 5 percent greater than the length of wild-type SV40. Yet Winocour et al. (1975) showed that the maximum length of virion DNA extracted from CV-1 cells infected with defective virions at high multiplicities of infection was 33 to 60 percent greater than wild-type SV40 DNA. Replicating SV40 DNA may be longer than wild-type length, but only DNA of a small range of lengths (70 to 105 percent wild-type length) can be packaged efficiently. Brockman



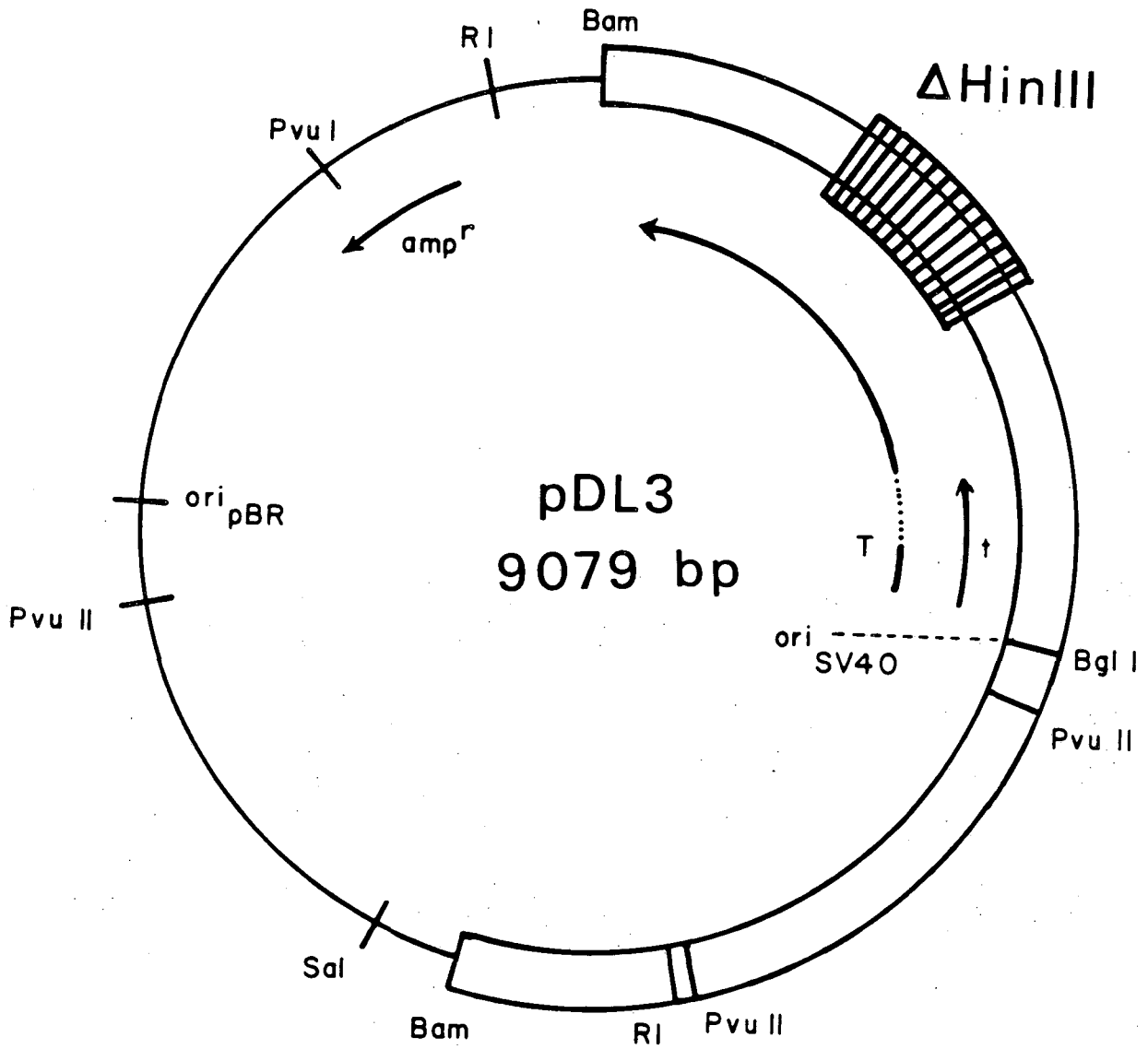
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Figure 1.1. Physical Maps of Plasmids ptsA-209 and pTK2.

and Nathans (1974) have reported an infectious variant of SV40 that has a one hundred base pair insertion. In the similar polyoma system, variants have been isolated that contain 4.5 to 5.0 percent more polyoma DNA than normal genome length (Fried and Griffin, 1977).

I decided to excise the SV40 late region, which codes for the virion coat proteins and insert in its place the HSV thymidine kinase gene. To produce an infectious virus, CV-1 cells would be cotransfected with the SV40-TK recombinant DNA and with the DNA from the SV40 deletion mutant, dl-1001 (Lai and Nathans, 1974). Dl-1001 contains a deletion of a Hind III restriction fragment in the SV40 early region. Dl-1001 cannot produce a functional T-antigen, but it can produce viral coat proteins and can replicate if supplied T-antigen in trans. Viral DNA of dl-1001 has been inserted into the plasmid vector pBR322, producing the plasmid pDL3, the physical map of which is shown in figure 1.2. A cotransfection of CV-1 cells with dl-1001 and the SV40-TK DNA was predicted to produce a mixed infection: the SV40-TK producing the T-antigen and dl-1001 producing the coat proteins needed by both viral DNAs to produce viral particles. In the proposed radiation studies, the dl-1001 virus would be "transparent" since it would not produce any functional T-antigen and could not transform rodent cells in vitro.

The herpes simplex viral thymidine kinase gene has been cloned (Enquist et al., 1979; Colbere-Garapin, et al., 1979) and sequenced (Wagner et al., 1981; McKnight, 1980). The HSV-TK plasmid, pTK2, that I used for these studies was a gift from Michael Botchan, and contains

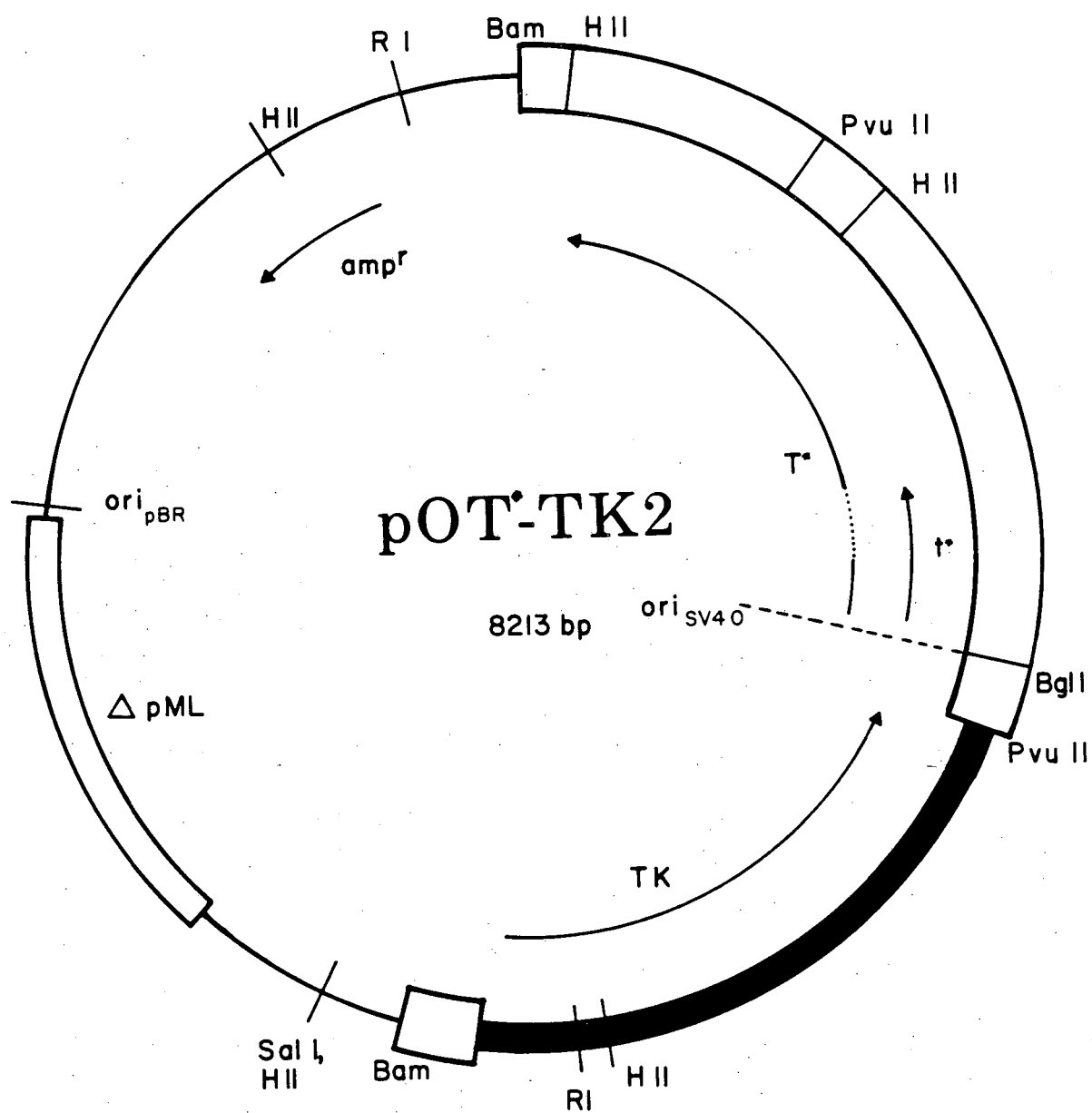


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Figure 1.2. Physical map of plasmid pDL3.

the 3.4 kbp Bam HI restriction fragment of TK (figure 1.1). By means of DNA mediated gene transfer, the 2025 base pair Pvu II restriction fragment has been shown to contain the intact TK gene by Colbere-Garapin and co-workers (1979). The final construction was plasmid pOT*-TK2 (figure 1.3; Appendix F), which contains the SV40 origin of replication, the tsA-209 A-gene, and the herpes simplex viral TK gene. If this plasmid is digested with Bam HI, a 5241 base-pair fragment containing the SV40-TK DNA is generated. SV40 wild-type length is 5243 base pairs; hence, the temperature sensitive mutant recombinant viral DNA is 99.96 percent wild-type SV40 length and should package efficiently.

There are two ways to study the biological properties of the recombinant SV40-TK DNA: plaque and replication assays. The most stringent approach is the plaque assay in which CV-1 cells are cotransfected with Bam HI digested pOT*-TK2 and pDL3 and then overlaid with agar. The formation of plaques, denoting a viral infection, would be scored in three weeks at 33°C (the permissive temperature of the mutant T-antigen). The replication assay follows the accumulation of replicating viral or plasmid DNA over a period of three days in the cell line COS-7. The COS-7 cell line is derived from the transfection of CV-1 cells with a SV40 DNA plasmid that contains a defective SV40 origin of replication (Gluzman, 1981). This cell line is SV40 A-gene transformed, synthesizes T-antigen, but produces no infectious virions. COS-7 supports the replication of SV40 temperature-sensitive A-gene



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Figure 1.3. Physical map of plasmid pOT*-TK2.

mutants (e.g., tsA-209) and SV40 A-gene deletion mutants (e.g., d1-10001). Under normal conditions SV40 virus cannot replicate in the CV-1 cells if it is cloned into the pBR322 plasmid vector. But Lusky and Botchan (1981) reported that a deletion derivative of pBR322, plasmid pML, can support the replication of SV40 cloned inserts. The plasmid pJYM contains the entire SV40 viral DNA inserted into pML, and can replicate in both CV-1 and COS-7 cells. POT*-TK2 also contains the pML vector and should replicate in COS-7 cells at temperatures from 33 to 39°C, since functional T-antigen is supplied by the COS-7 cell.

MATERIALS AND METHODS

For the sake of convenience and efficiency, the reagents and procedures for molecular cloning, mammalian cell culture, mammalian DNA analyses, and DNA manipulations are presented in the Appendices. The details of each technique will be found by referring to the cited Appendix.

Cells and Culture Techniques. The cell lines CV-1 and COS-7 were obtained from Michael Botchan (App.I.II). Cultures were grown in Dulbecco's Modified Eagle medium (DME; Gibco), containing 10 percent fetal calf serum (Gibco), 100 IU/ml penicillin (Gibco), and 50 µg/l streptomycin (Gibco) (App.B.IV).

Plasmid DNA. The construction of pOT*-TK2 and ptsA-209 is described in Appendix F. Plasmids pDL3 and pJYM were a gift from Monika Lusky (Appen. F). All plasmids were grown by the modification of the procedure by Birboim and Doly, (1979) (App.D.I).

Viral DNA. Wild-type SV40 and SV40 mutant tsA-209 viral DNA were prepared from infected CV-1 monolayers in 100 mm dishes. Cells infected with tsA-209 were incubated at 33°C, and cells infected with SV40 were incubated at 37°C. The infections were with multiplicities greater than unity. Viral DNA was prepared by the procedure of Hirt (1967) as modified by Sambrook (1972) (Appen.D.II.B).

Plaque Assay. Sub-confluent CV-1 cells were co-transfected (via DEAE Dextran) with Bam HI digested pDL3 and pOT*-TK2 DNAs. One microgram of each DNA was transfected per 60 mm dish. Bam HI digested SV40 viral DNA was transfected as a control (1 ng/60 mm dish). After transfection, the cell monolayers were overlaid with agar as described in Appendix H.I.

Replication Assay. Subconfluent COS-7 cells in 60 mm dishes were transfected via DEAE-Dextran (App.H.I) with SV40 viral DNA, tsA-209 viral DNA, pJYM, and pOT*-TK2. Cells were incubated at 37°C. Low molecular weight DNA was extracted by the procedure of Hirt (1967) (Appen. D.II) at time periods 0, 24, and 48 hours post-transfection. The Hirt pellets (containing the low molecular weight DNA) was suspended in gel loading buffer and electrophoresed through 1.0 percent agarose gel and transferred to nitrocellulose filter by the methods of Southern (1975; App.G). Labeled SV40 DNA was used to probe the nitrocellulose filter (App.C.III).

RESULTS

Plaque Assay. Only four plaques were scored on four total dishes. These plaques were isolated and used to infect fresh CV-1 monolayers. Restriction analysis of Hirt lysates demonstrated rearrangements of the recombinant DNA, particularly showing deletions in the promoter regions of the thymidine kinase gene (data not shown).

Replication assay. Figure 1.4 shows the autoradiogram of the Southern blot (Appendix G) of the replication assay. Panel A represents 10 ng of pJY1 DNA (9.6 kbp, Appen. F) as a transfer and hybridization control. Panels b-e represent the extent of replication of viral SV40, viral tsa-209, plasmid pJYM, and plasmic pOT*-TK2 DNAs. In each panel the input DNA at the 0-hr time point is just discernible. At 24 and 48 hours post-transfection, both viral DNAs and plasmic pJYM replicate, but no detectable replication of pOT*-TK2 can be seen. The second band seen in panel c denotes the accumulation of defective viral DNA that is normally observed with mutant tsA-209 production.

The inability of pOT*-TK2 to replicate is not due to its bacterial component, since SV40 DNA inserted into the same vector replicates very well. The deletions and rearrangements in the promoter region of the HSV TK gene suggested that there might be some form of biological incompatibility between the HSV TK gene and SV40 replication. I decided to dissect the regions of DNA within plasmid pOT*-TK2 that "poisons" replication. My approach was to perform a replication assay with pOT*-TK2 DNA that had been digested with

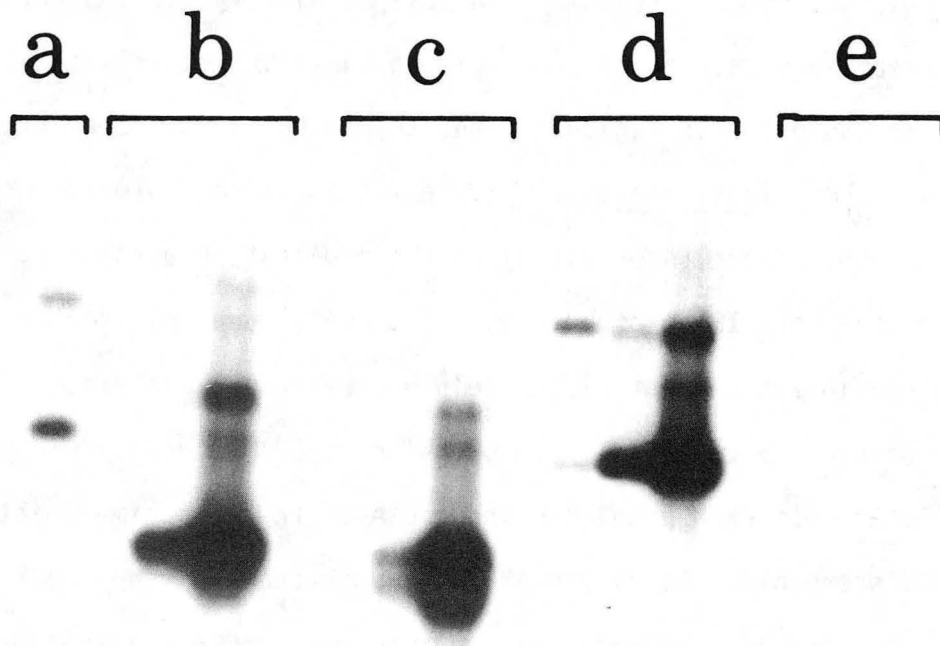
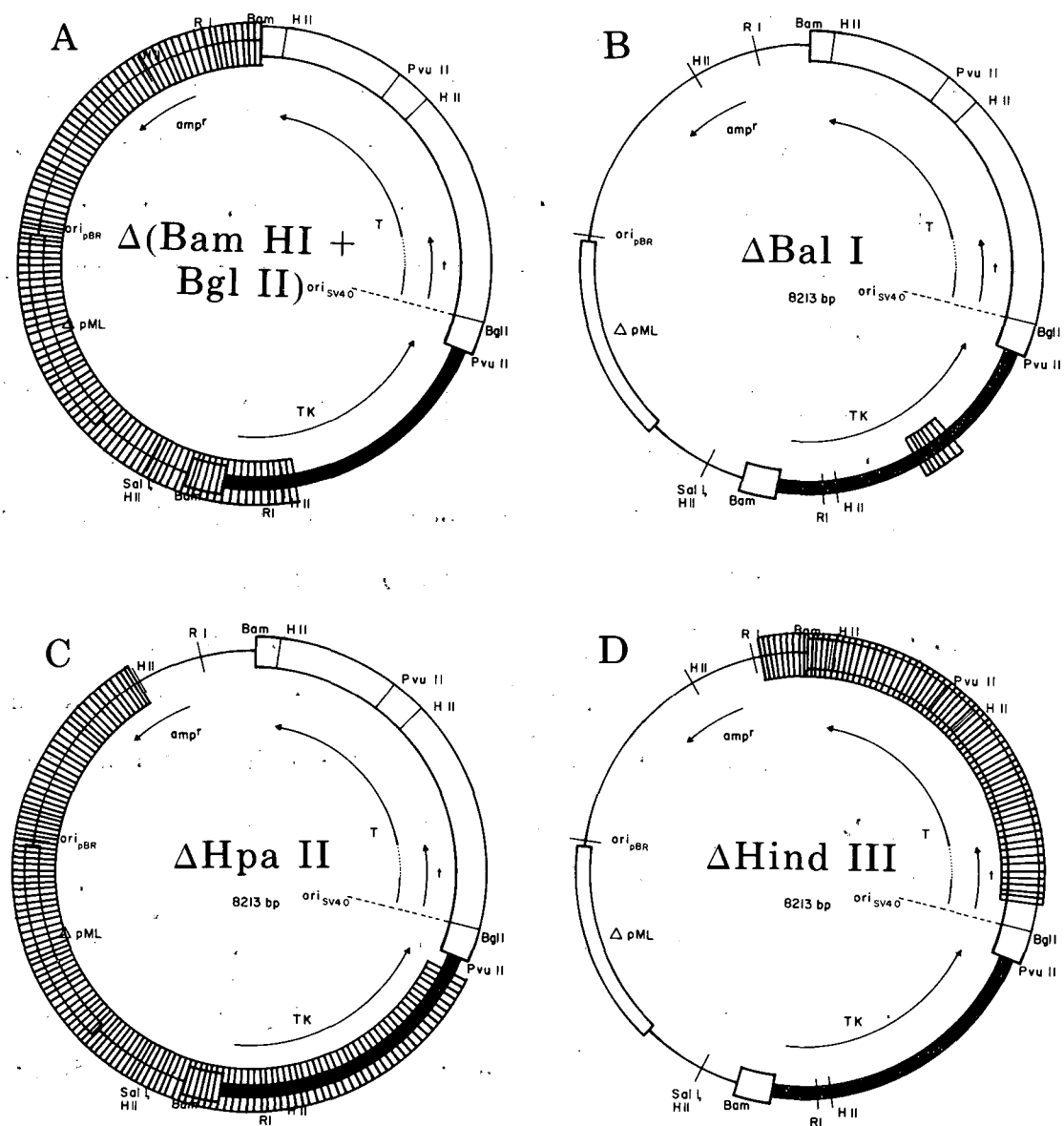


Figure 1.4. Replication Assay in COS-7 cells.

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- Panel a: 10 ng uncut pJY1 DNA
- Panel b: Wild-type SV40 viral DNA
- Panel c: tsA-209 viral DNA
- Panel d: plasmid pJYM DNA
- Panel e: plasmid pOT*-TK2 DNA



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Figure 1.5. Schematic diagram of endonuclease cleavage "deletions."

- Panel A: Bam HI + Bgl II -- TK-promoter deletion
 Panel B: Bal I -- Partial TK-gene deletion
 Panel C: Hpa II -- Complete TK-gene deletion
 Panel D: Hind III -- SV40 A-gene deletion

specific restriction endonucleases. Upon transfection into COS-7 cells, the restriction fragment would recircularize by cellular ligation, and the T-antigen necessary for replication would be supplied in trans by COS-7. Figure 1.5 schematically portrays these deletions (represented by the hatched regions) generated by the restriction endonuclease digestions. The strategy employed involved the following:

- A. TK-promoter deletion. Double digestion with Bam HI and Bgl II liberates the TK promoter from the HSV TK-gene (Wagner et al., 1981; McKnight, 1980) and produces cohesive DNA termini that allow for cellular mediated recircularization.
- B. Partial TK-gene deletion. Bal I digestion produces a substantial deletion within the TK structural gene. I wanted to explore the idea that the thymidine kinase enzyme itself is responsible for the replication poison.
- C. Complete TK-gene deletion. Hpa II digestion deletes the entire thymidine kinase gene and acts as a replication control, ensuring that the SV40 origin of replication had not been functionally mutated during the genetic engineering of pOT*-Tk2.
- D. SV40 A-gene deletion. Hind III digestion deletes the SV40 A-gene but leaves an intact SV40 origin of replication covalently linked to an intact thymidine kinase gene. I wanted to test the possibility that both A-gene and TK-gene transcription interfere with pOT*-TK2 replication.

MATERIALS AND METHODS

DNA digestions. One microgram of pOT*-TK2 was digested to completion in 50 ul with each of these enzymes: Bam HI + Bgl II, Bal I, Hpa II, and Hind III.

Replication Assay. 10 ng of each digested plasmid DNA and uncut viral SV40 DNA was transfected per 60 mm dish of sub-confluent COS-7 cells as described previously. Time points for Hirt extraction were 0, 24, and 48 hours post-transfection. The nitrocellulose filter was hybridized with labeled pOT*-TK2 DNA.

RESULTS

Figure 1.6 represents the autoradiogram of the replication assay. Panel a depicts 10 ng of uncut pOT*-TK2 DNA as a transfer and hybridization control. Panel b shows the replication of uncut SV40 viral DNA; panels c-f, the replication of pOT*-TK2 DNA digested with (Bam HI + Bgl II), Bal I, Hpa II, and Hind III respectively. With the promoter deleted, the recombinant SV40-TK DNA is able to replicate a small amount, but not at the efficiencies seen with the TK gene completely deleted (SV40 viral DNA or the Hpa II digest). Neither a small deletion in the TK structural gene (Bal I digestion) nor complete deletion of the SV40 A-gene (Hind III digestion) support replication. (The SV40 origin of replication is capable of replicating in COS-7 cells, data not shown.)

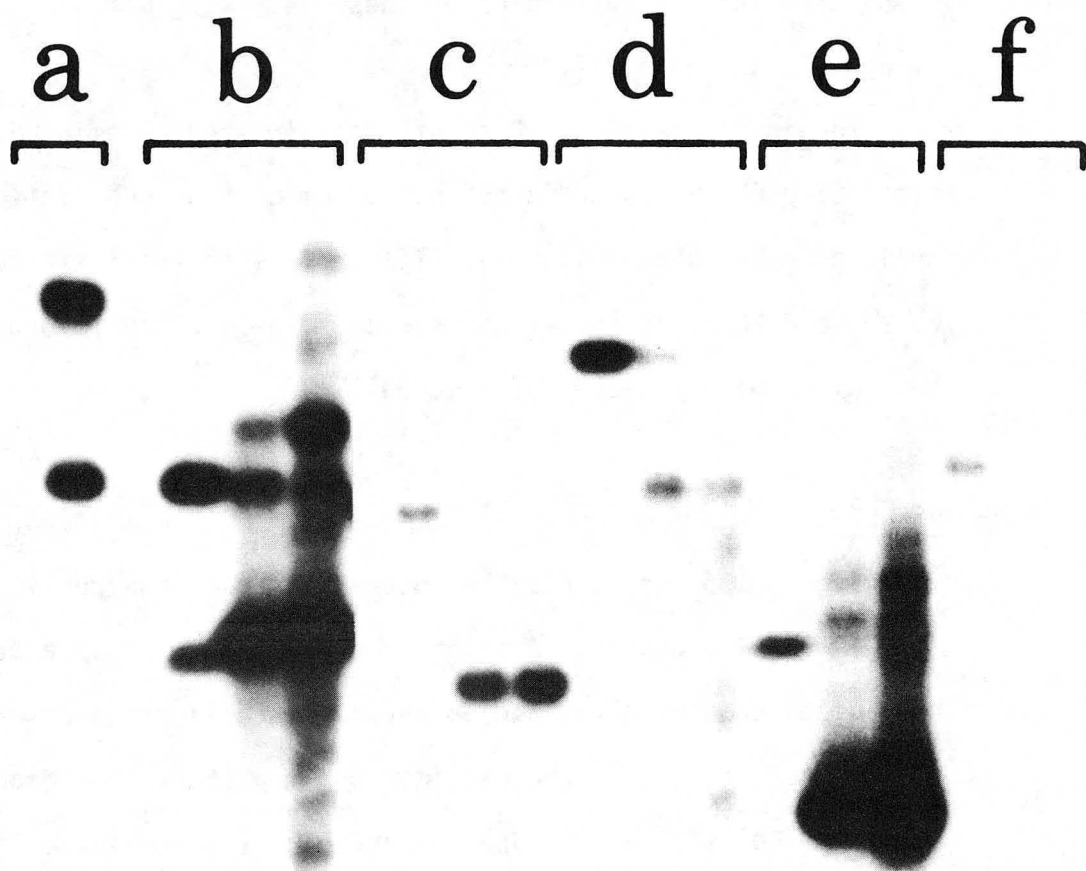


Figure 1.6. Replication Assay in COS-7 cells.

Panel a: 10 ng uncut pOT*-TK2 DNA

Panel b: Wild-type SV40 viral DNA

Panel c: pOT*-TK2 DNA digested by Bam HI + Bgl II

Panel d: pOT*-TK2 DNA digested by Bal I

Panel e: pOT*-TK2 DNA digested by Hpa II

Panel f: pOT*-TK2 DNA digested by Hind III

DISCUSSION

The plaque and replication assays strongly suggest that thymidine kinase transcription is incompatible for SV40 replication in simian cells. An intact HSV TK gene or a deleted TK gene still "poisons" replication, while the deletion of the TK promoter allows for some replication. In the case of the promoter deletion, it is possible that the thymidine kinase gene is being transcribed by the SV40 A-gene promoter. This inefficient transcription might allow for a small amount of replication—a hypothesis not without merit, since a polyoma-TK recombinant virus has been produced (Dinces and Milman, 1982) that mirrors the construction of pOT*-TK2. In this construction the HSV TK gene promoter has been deleted by Bgl II digestion with the TK gene expression mediated by the polyoma early promoter, which is 2500 base pairs away from the TK structural gene. Such a result also suggests that the polyoma virus can replicate despite TK transcription in mouse cells, but this is not the case with SV40-TK recombinant DNA in simian cells.

Paul Robbins from Michael Botchan's laboratory has also studied the effects of TK-transcription upon the replication of recombinant SV40 plasmids (personal communication). He constructed HSV-TK plasmids with an SV40 origin of replication with and without SV40 sequences that increase the efficiency of gene expressions ("enhancers", reviewed in Khoury and Gruss, 1983). Plasmids that were more efficient in TK gene transcription replicated an order of magnitude less than plasmids that were not efficiently transcribing the TK gene.

At that juncture in time, I thought it highly improbable to rescue a recombinant SV40-TK virus because of the aforementioned data. I decided to pursue the effects of radiation on the efficiency of DNA mediated gene transfer (Chapters 2, 3, and 4). The results of this work constitutes the rest of this dissertation.

Even without the SV40-TK virus I have been able to study the effect of ionizing and non-ionizing radiation on efficiency of DNA mediate gene transfer with various recombinant DNA plasmids. The next chapters present these findings.

CONCLUSION

A recombinant SV40-TK DNA plasmid was constructed but a recombinant SV40-TK virus could not be rescued due to the inability of the recombinant DNA to replicate in simian cells.

CHAPTER TWORADIATION ENHANCES THE EFFICIENCY OF DNA MEDIATED GENE TRANSFER

My original objective was to investigate the phenomenon of radiation enhancement of SV40 transformation. Did SV40's unique biology allow for its own enhancement? The proposed recombinant SV40-TK virus would provide two biological parameters: a thymidine kinase gene marker and a temperature sensitive T-antigen. I hoped to study T-antigen's role in the radiation enhancement of transformation by modulating the temperature of the cell culture conditions. Using the TK gene as a marker, I planned to study the presumed initiation step of transformation—integration of the viral DNA. Chapter One revealed the impasse I faced: produce a recombinant virus with a non-functional TK gene or be left with functional recombinant plasmid DNA that would not form a virus.

I decided to choose the latter condition and introduce the recombinant plasmid into the cell by DNA mediated gene transfer (DMGT) and then irradiate the cells. Over 22 years ago, Syzbalska and Szybalski (1962) first reported the DNA mediated transfer of the gene coding for the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) into HGPRT⁻ (called at that time IMPPase⁻) mutant human cells. DNA mediated gene transfer (also called DNA transfection) was not reproducible for years because researchers were unable to appreciate the role of the calcium phosphate precipitation for producing a successful transformation. The phosphate was present in the normal culture media and the calcium was provided in the

particular spermine preparation in which Szybalska and Szybalski stored their DNA. Only a decade later did Graham and Van der Eb (1973) develop the reproducible procedure to facilitate DNA mediated gene transfer. By their methodology, DNA is added to a buffered phosphate solution, which was then mixed with a solution of calcium chloride. The resulting precipitate, which contained the DNA as well, was added directly to the tissue culture cells.

There is one major difference between transferring genes via viral infection versus DNA transfection. In a viral infection, the viral DNA has been packaged into a particle by its host cell and is protected from the recipient cell's digestive enzymes, whereas by the transfection protocol the DNA is complexed with calcium phosphate and enters the cell as 'food' (see Chapter Four) open to nuclease attack. My initial concern was that DNA transfection might produce many strand breaks from the shearing experienced through all the in vitro manipulations, which in turn could interfere with the integration process. On the other hand, without the packaging restraint imposed by SV40, any size gene marker could be linked to the SV40 A-gene. More importantly, since all plasmid DNA would be produced in bacterial cells, the role of SV40 sequences in any observed enhancement would be studied by merely deleting them from the recombinant plasmid. The stage was indeed set for an exciting study.

FOR NUMBERING SEQUENCE ONLY

MATERIALS AND METHODS

Cells and Culture Techniques

The cell line Rat-2 (Topp, 1981) was obtained from Michael Botchan. Rat-2 is thymidine kinase deficient (tk^-), flat, and nonpermissive to SV40 viral infection. Stock cultures were grown in Dulbecco's Modified Eagle medium (DME), containing 10 percent fetal calf serum (Gibco), 100 IU/ml penicillin, 50 μ g/ml streptomycin (Gibco), and 100 μ g/ml 5-Bromodeoxyuridine (Sigma). The cells were kept in a humidified Wedo incubator with an atmosphere of 90 percent air + 10 percent CO_2 at 37°C. CV-1 cells were grown in DME in 10 percent FCS.

SV40 DNA

The preparation of SV40 viral DNA is described in Chapter One.

Plasmid DNA

The details of the construction of plasmids pTK2 (Figure 2.2) and pOT-TK5 are presented in Appendix F. Plasmid pOT-TK5 contains the intact wild-type A-gene and origin of replication and the intact Herpes simplex virus type 1 thymidine kinase gene (Figure 2.3).

Plasmid DNA was isolated as described in Chapter One.

DNA-Mediated Gene Transfer

Stock cultures were passaged once in DME without BUdR, then plated into plastic dishes. Confluent monolayer cells were used at a density of 7.0×10^4 cells/cm². The donor material—either SV40 viral DNA, pTK2, or pOT-TK5—was isolated as Form I DNA and was composed of a mixture of monomers and multimers. Precipitation and transfection of

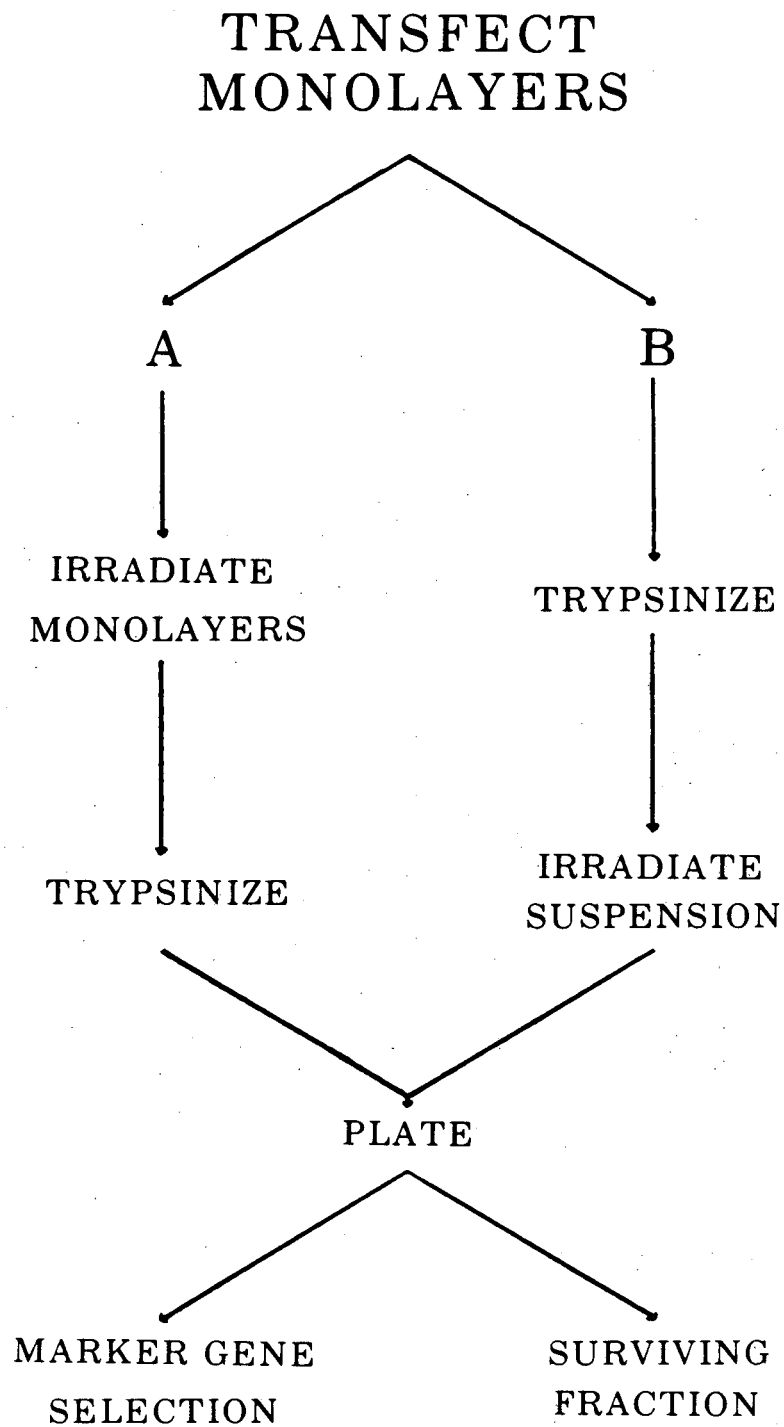
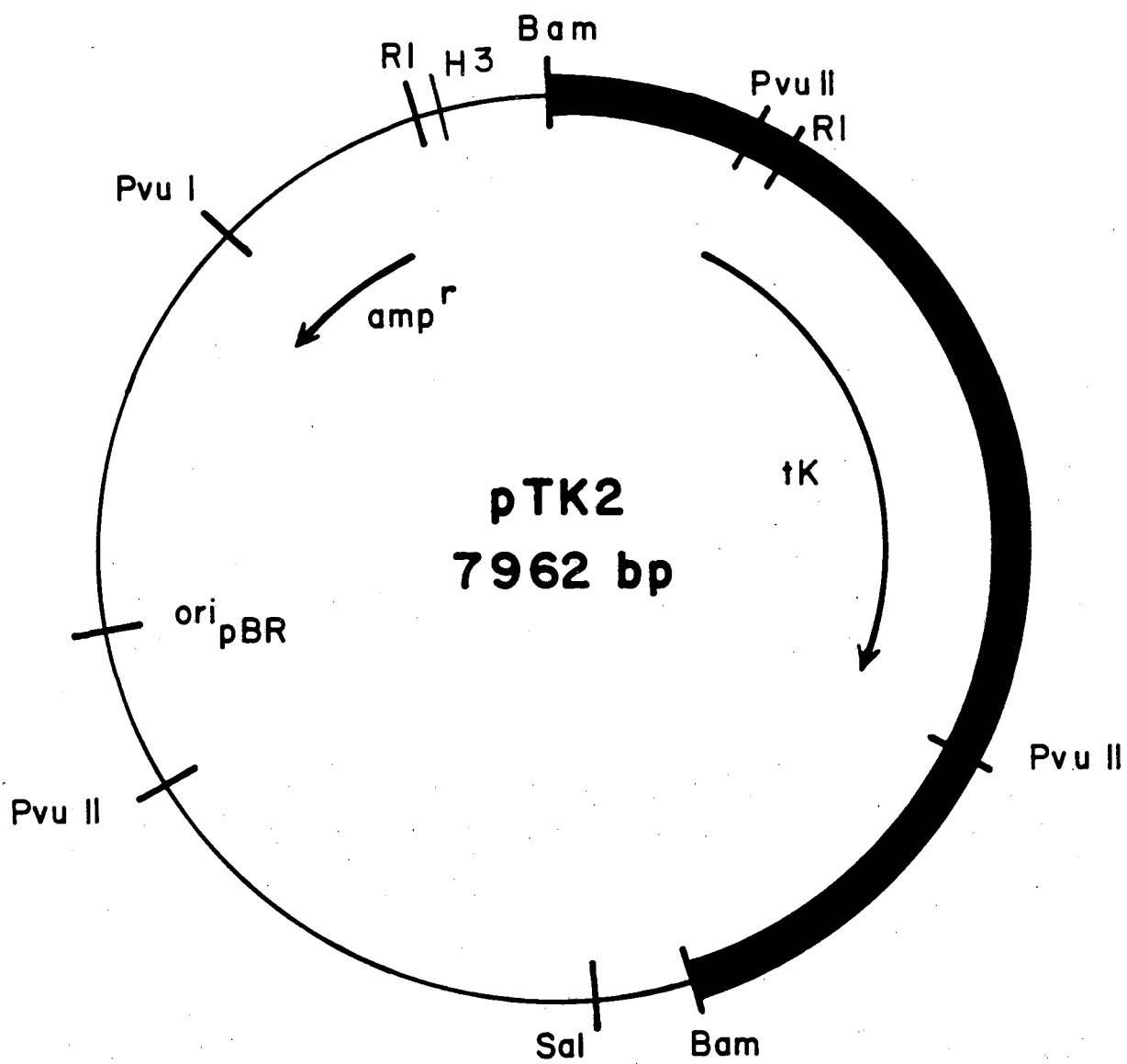
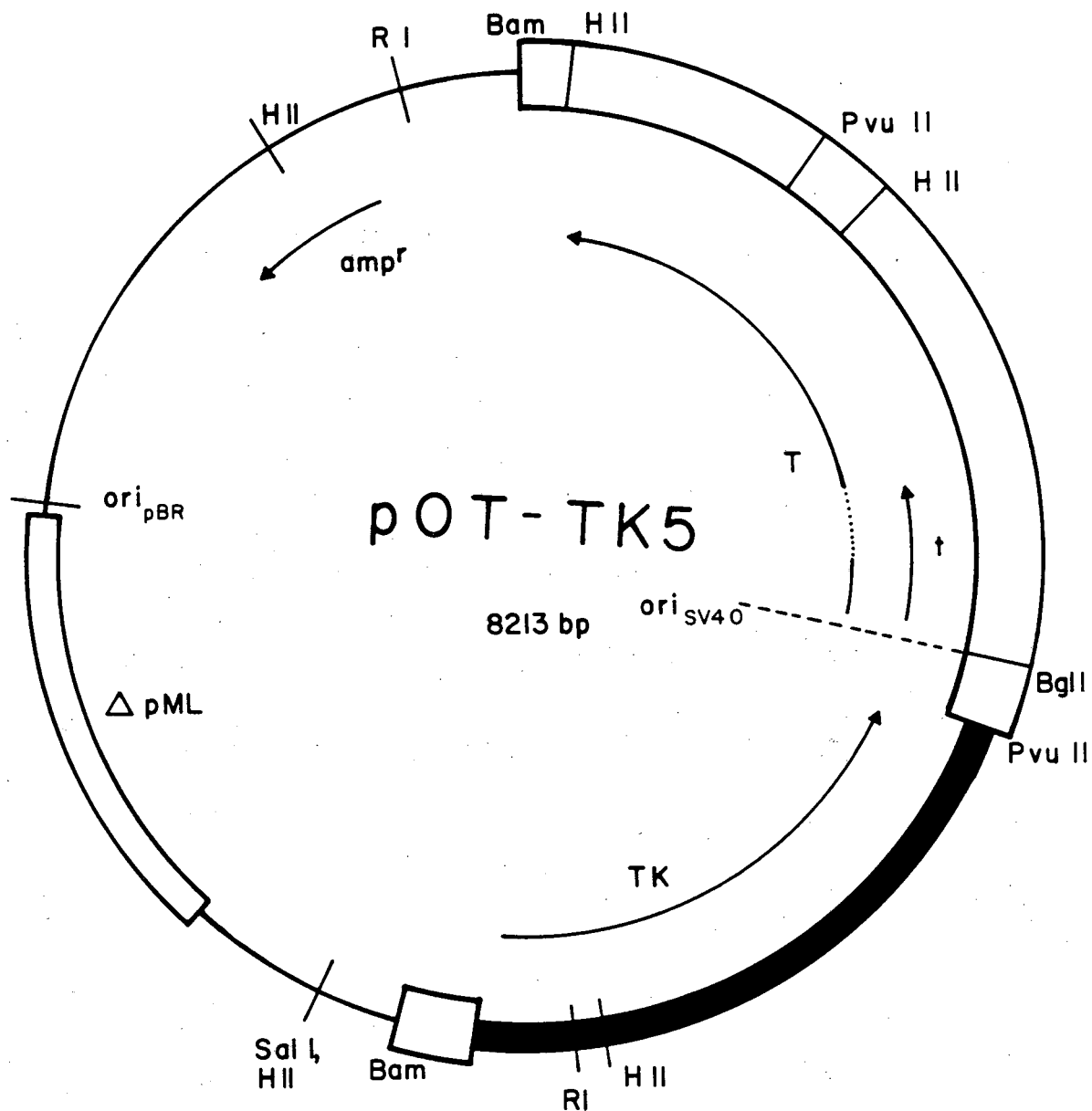


Figure 2.1. Schematic Diagram of Radiation Enhancement of DNA Mediated Gene Transfer Experiments.



XBL 836-10417

Figure 2.2 Physical map of plasmid pTK2.



XBL 836-10418

Figure 2.3 Physical map of plasmid pOT-TK5.

DNA are described in detail in Appendix H. The vessels containing DNA precipitate were incubated at 37°C. The medium with precipitate was removed from the dishes after twelve hours, and fresh medium was added. At twelve hours post-DNA treatment, cells were prepared for irradiation (Figure 2.1).

Irradiation

X rays. Irradiations were done with a Philips 250-kVp X-ray Unit. Physical conditions of irradiation were 225 kVp, 15 mA, and half-value layer of 1.1 mm of copper. Dose rates of 85 and 55 rad/min were used for the cell suspension and monolayer experiments, respectively, as measured with a Victoreen condenser R meter. Corrections were made for temperature, atmospheric pressure, and the dosimeter calibration factor. A constant factor of 0.95 was used to convert Roentgen into rad. Cells prepared for suspension irradiation were removed from culture dishes with an EDTA-trypsin solution, and a suspension with a density of 1.5×10^6 cells/ml medium was made. 10 ml of the cell suspension were placed in a cylindrical suspension chamber (3 cm in diameter and 1.5 cm thick) and irradiated at room temperature, being stirred continuously with a small Teflon-coated magnetic bar. Cells were diluted in medium and plated for HAT selection and colony forming ability (CFA).

Cells irradiated in monolayers in dishes were trypsinized, counted, and plated for HAT selection and CFA.

Heavy-Ion Irradiation. A monoenergetic argon beam generated by the BEVALAC with an initial energy of 330 MeV/amu at the exit window

was used for this experiment. The average LET of argon particles at the sample position was 144 keV/ μm . Cells were irradiated in suspension and plated identically to the X-ray irradiation techniques.

In all experiments, irradiation of cells was performed at room temperature. The X-ray and argon experiments were done within one hour of each other, with cells originally obtained from the same population. Ten days after irradiation CFA dishes were fixed with glutaraldehyde and stained with methylene blue.

HAT Selection

Forty-eight hours after plating, HAT selection was initiated by changing medium with DME supplemented with 5 percent FCS, 100 μM Hypoxanthine, 1 μM Aminopterin, and 40 μM Thymidine (Sigma). The cells were fed every two to three days, and colonies were fixed and stained after ten days of selection.

Assay for SV40 A-gene Expression

Approximately 10^5 viable cells were plated in 100 mm dishes in DME with 10 percent FCS. Monolayers were fed once a week with DME, 5 percent FCS. Twenty-one days after irradiation, monolayers were fixed, stained, and scored for piling up foci.

Calculation of Enhancement Ratio

The enhancement ratio for marker gene transformation was calculated as follows:

$$\text{Enhancement Ratio} = \frac{(\text{Transformants per survivor})_{\text{irradiated}}}{(\text{Transformants per survivor})_{\text{unirradiated}}}$$

Error bars were calculated to 95 percent confidence intervals.

RESULTS AND DISCUSSION

Actual data from all experiments is tabulated in Appendix A. The specific data for each experiment will be cited during the discussion.

Ionizing radiation sensitivity of Rat-2 cells. Rat-2 cells displayed normal sensitivity to graded doses of 225 kVp X-rays and 330 MeV/amu argon ions (Figure 2.4; A2, A3). The Bragg curve for this experiment is in Appendix J. From traditional target theory, the slopes of the final straight portion of the survival curves, D_0 's, were 1.20 Grays for X-rays and 0.57 Gray for the argon ions. The extrapolation number, n , was found by extrapolating the straight portion of the survival curve until it crossed the axis of the "surviving fraction." The extrapolation number for both X-rays and for argon ions was 43. The common extrapolation number suggests that despite the high LET measurement (144 keV/ μ m) the plateau 330 MeV/amu argon ions are producing more ionization but not at saturating densities.

The differences in the two survival curves can also be explained by the Repair-Misrepair Model (Tobias et al., 1980). By this model the argon ions are producing more ionizations per unit distance along the track than X-rays, which in turn produces more lesions. The cell is unable to repair all the lesions without producing a higher frequency of errors--leading to misrepair and consequently more cell killing.

Further studies are needed to delineate the effects of radiation quality on cell killing with the Rat-2 cell line.

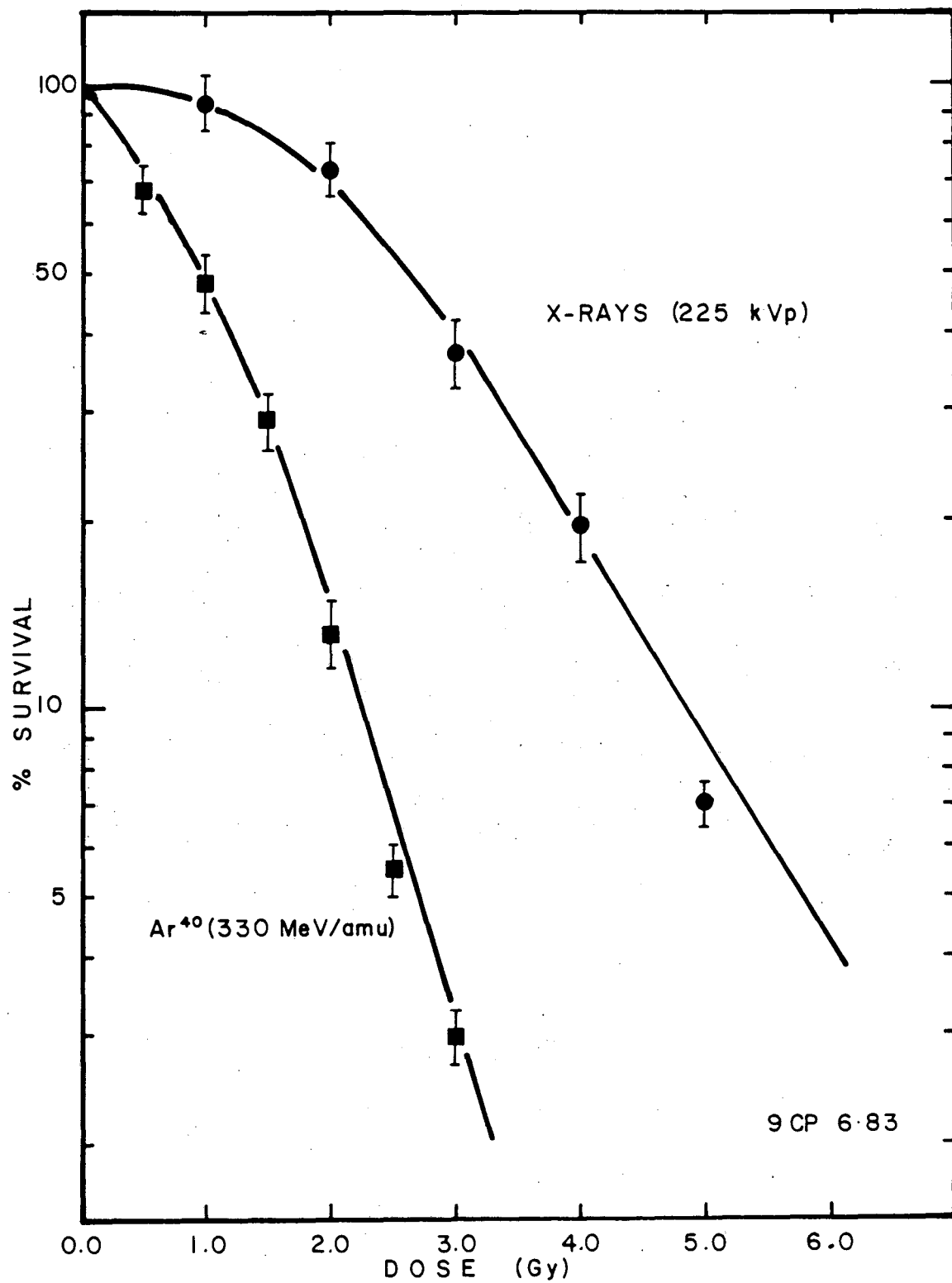


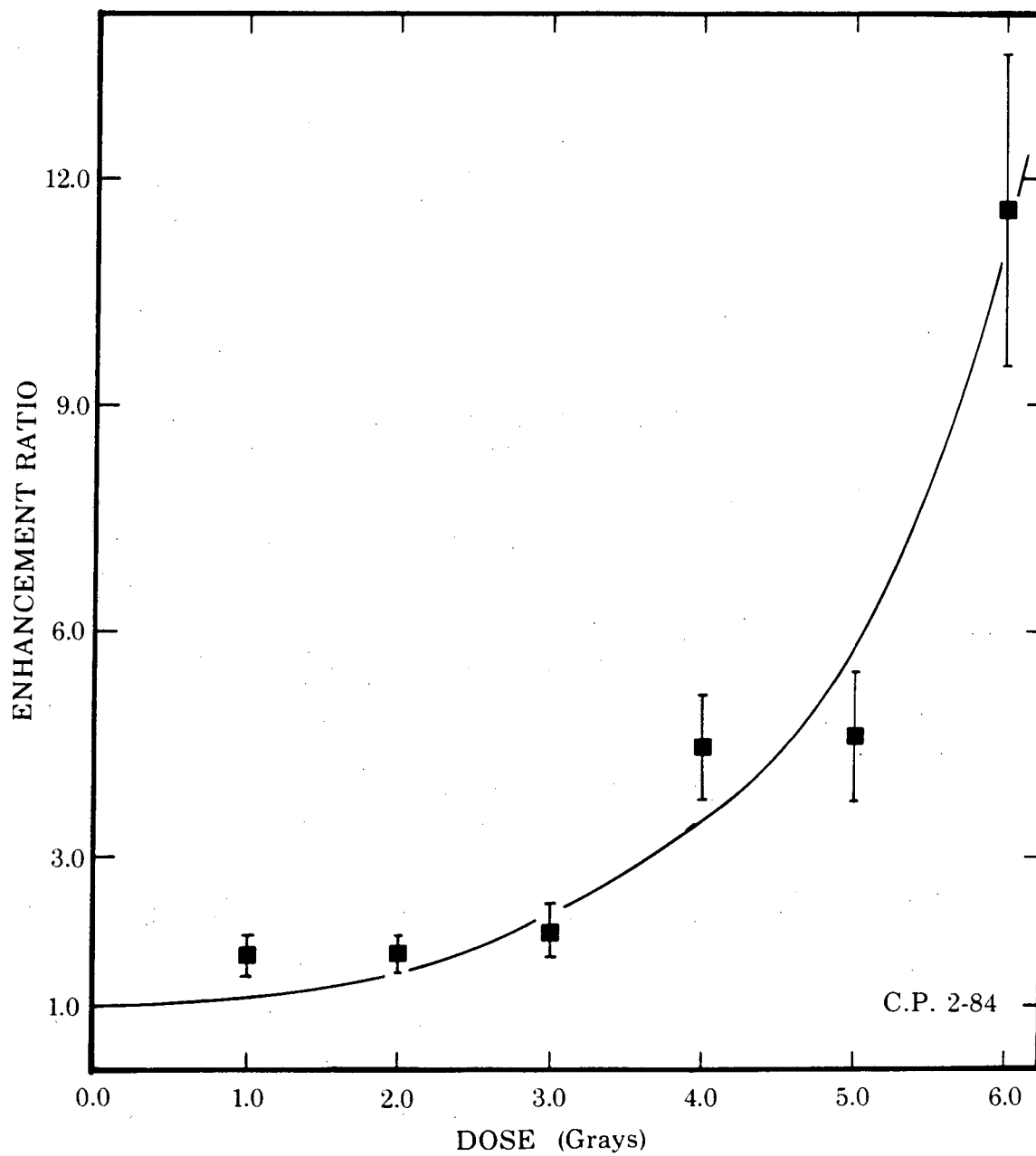
Figure 2.4. Survival curves of High and Low LET ionizing radiation for Rat-2 cells.

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Ionizing radiation enhances A-gene transformation in SV40 transfected cells. Rat-2 cells transfected with SV40 viral DNA were irradiated with graded doses of 225 kVp X-rays. X-rays enhanced A-gene transformation (Figure 2.5). This enhancement was non-linear and dose dependent, which is similar to the radiation enhancement observed with virally infected $10T_{1/2}$ cells (Yang et al., 1980).

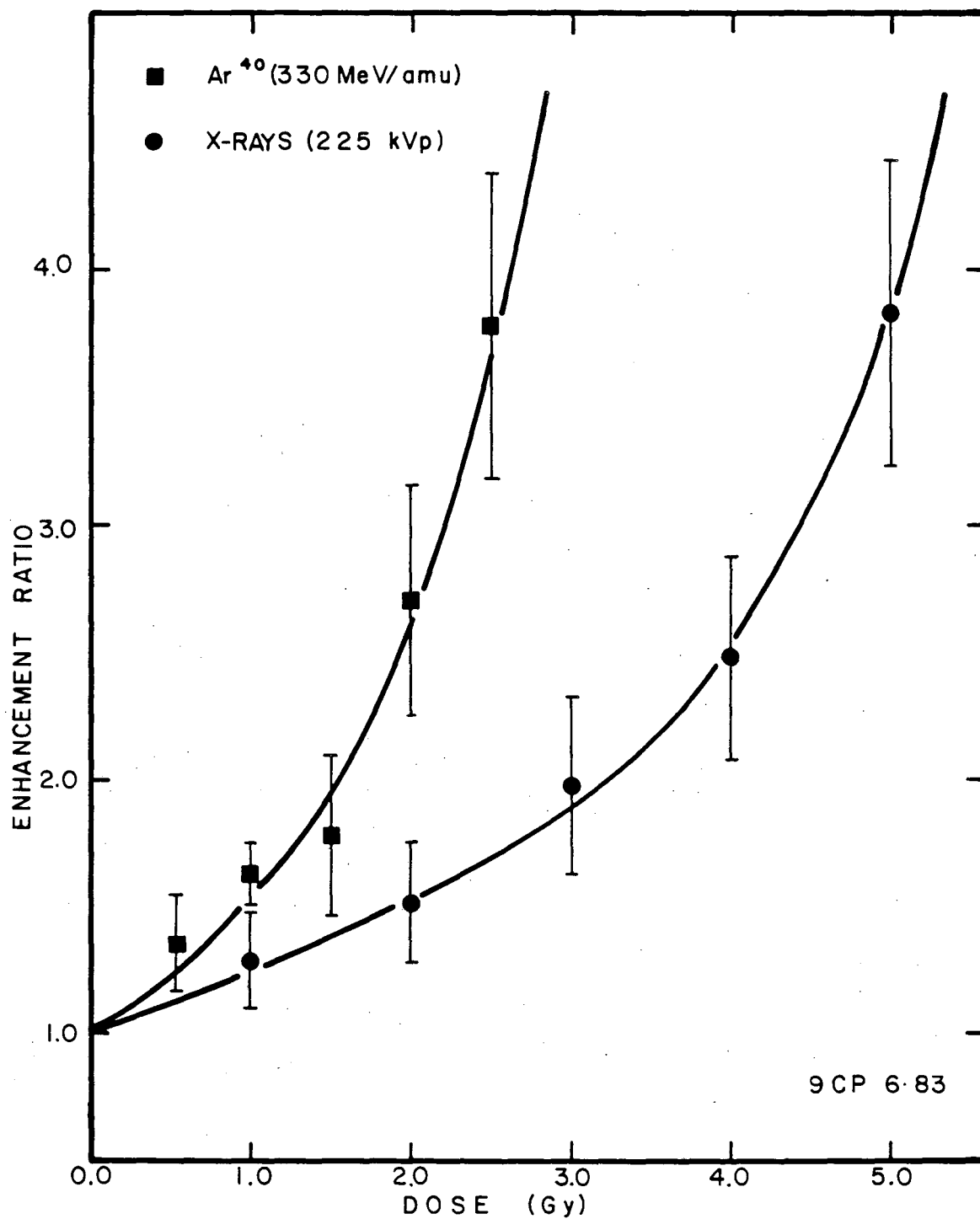
Ionizing radiation enhances TK-transformation in pOT-TK5 transfected cells. Rat-2 cells were transfected with pOT-TK5 and were irradiated with graded doses of X-rays and 330 MeV/amu argon ions. In both cases the efficiency of TK-transformation was increased, with argon ions more efficient than X-rays in enhancing this transformation (Figure 2.6; A2, A3). The RBE for enhancement was 2.0 ± 0.5 for all surviving fractions. This effect is not surprising considering that cell killing with these argon ions may be dose-modifying, but further analyses may show different effects. The actual measured yield of HAT^+ colonies per cell plated was not substantially greater than the unirradiated transfected cultures (Figure 2.7; A2), but the yield curve was significantly greater than the calculated curve for no radiation enhancement.

Ionizing radiation enhances A-gene transformation in pOT-TK5 transfected cells. The kinetics of X-ray enhancement of TK-transformation with pOT-TK5 seemed similar to that of A-gene transformation with viral SV40 DNA. Would enhancement of both markers be the same if linked on the same plasmid Cells were transfected as



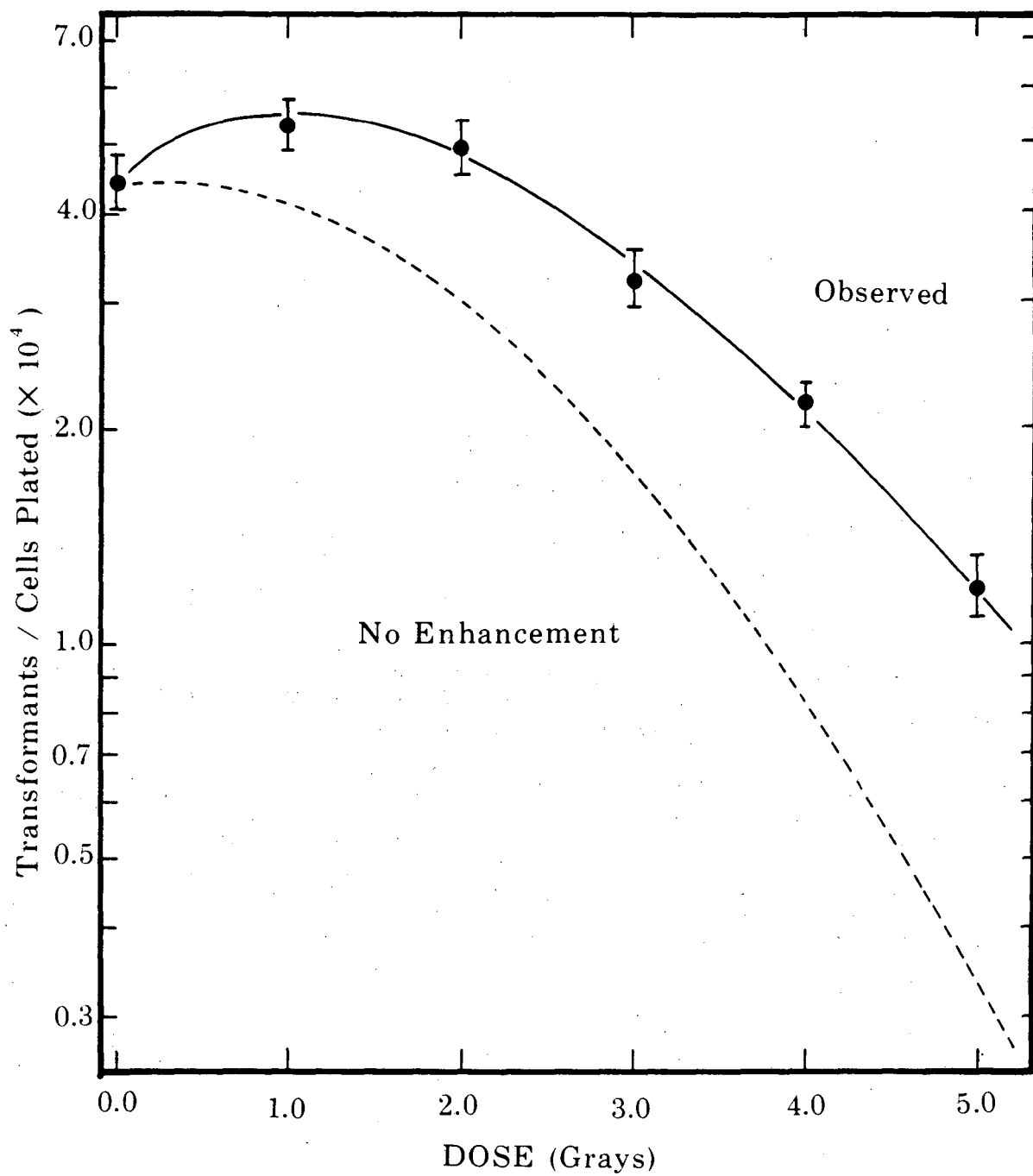
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Figure 2.5. X-ray enhancement of A-gene transformation of SV40 DNA transfected Rat-2 cells.



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Figure 2.6. Ionizing radiation enhancement of TK-transformation of pOT-TK5 transfected Rat-2 cells.



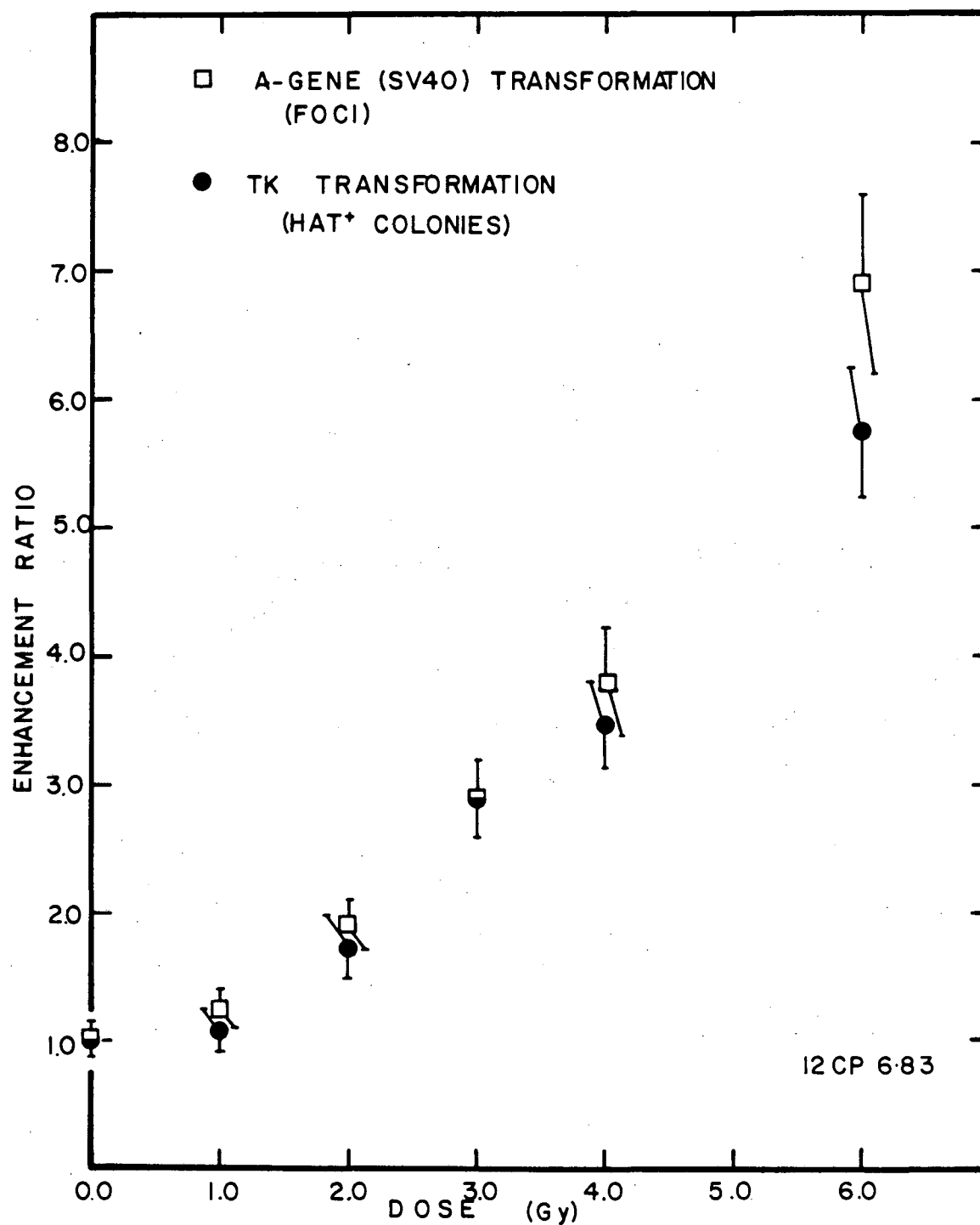
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Figure 2.7. X-ray dose response for the frequency of HAT⁺ transformants per cell plated of pOT-TK5 transfected Rat-2 cells.

before with pOT-TK5, trypsinized, irradiated in suspension with graded doses of X-rays, and plated. One set of cultures was selected in HAT media for TK-transformation and the duplicate cultures were selected for piling-up foci in DME without HAT for A-gene transformation. The radiation enhancements of these separately selected markers from the same transfected Rat-2 cells appear to be identical (Figure 2.8; A4, A5). Though the enhancement induction appears to be identical, the efficiency of gene expression was not. At 0.00 Grays the efficiency of TK-transformation was 31.9×10^{-4} transformants per survivor, compared to 4.61×10^{-4} transformants per survivor for A-gene transformation. For every A-gene transformant there were approximately 7 TK-transformants.

Co-transformation experiments argue for a random integration of pOT-TK5 in Rat-2 cells. I allowed one set of HAT-selection cultures to continue to incubate for two weeks to allow for the formation of piling-up foci (A-gene transformants). I observed that of 100 HAT⁺ clones only 12 were also A-gene transformants. Similarly, I found that of 50 A-gene transformed clones 42 were able to survive in HAT media. I utilized the conditional probability relation, $p(C|D) = p(C|D)p(D)$, to determine the frequency of co-transformation, $p(A|TK)$ by both experiments,

$$\begin{aligned}
 p(A|TK) &= p(A|TK)p(TK) \\
 &= (0.12 \pm 0.06)(3.19 \pm 0.18) \times 10^{-3} \\
 &= (3.8 \pm 1.9) \times 10^{-4}, \text{ for experiment one.}
 \end{aligned}$$



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Figure 2.8. X-ray enhancement of A-gene and TK-gene transformation of p0T-TK5 transfected Rat-2 cells. Replicate cultures selected for HAT⁺ colonies and piling-up foci.

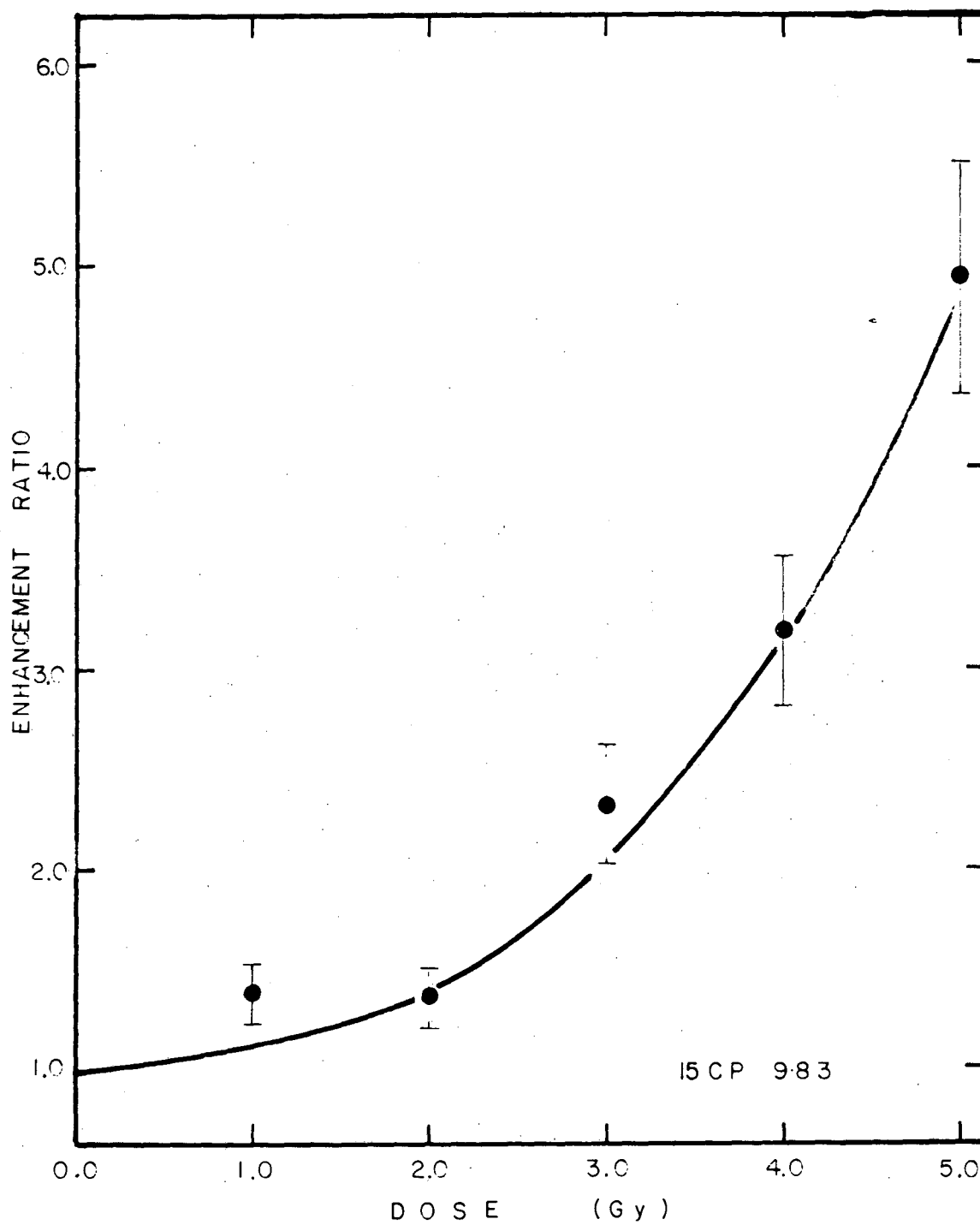
$$\begin{aligned}
 p(A|TK) &= p(TK|A)p(A) \\
 &= (0.84 \pm 0.13)(4.61 \pm 0.31) \times 10^{-4} \\
 &= (3.9 \pm 0.7) \times 10^{-4}, \text{ for experiment two.}
 \end{aligned}$$

Within 95 percent confidence the co-transformation frequencies are the same, therefore pOT-TK5 integration is random.

Radiation enhances TK-transformation without linked SV40 sequences.

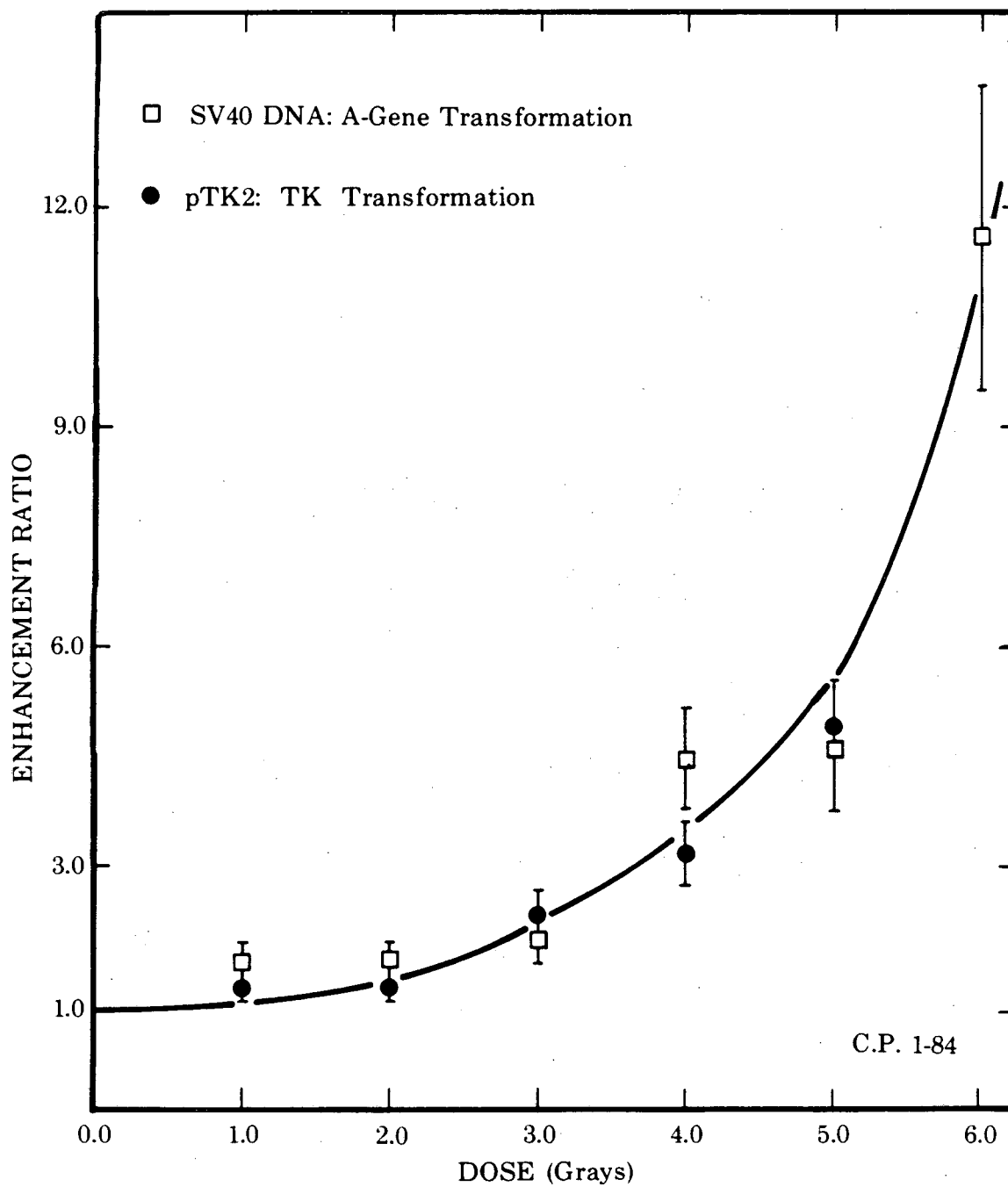
From the previous experiments it was clear that radiation enhanced the frequency of two gene markers: SV40 A-gene and HSV TK-gene. But the TK-transformation enhancement of pOT-TK5 transfected Rat-2 cells might be affected by the linked SV40 sequences coding for the SV40 tumor antigens and/or the SV40 enhancer sequence (Khoury and Gruss, 1983; for a review). Rat-2 cells were transfected with pTK2, which contains no SV40 sequences, and irradiated with graded doses of X-rays. X-rays enhanced TK-transformation of pTK2 transfected Rat-2 cells (Fig. 2.9), and was not significantly different from the A-gene enhancement of SV40 viral DNA transfected cells (Fig. 2.10; A1, A6). Radiation enhancement is a general cellular phenomenon and is not associated with either SV40 A-gene or enhancer function.

Ultraviolet radiation enhances the efficiency of DNA mediated gene transfer. Transfected Rat-2 cells show normal sensitivity to graded doses of ultraviolet radiation (Fig. 2.11). Ultraviolet radiation enhanced TK-transformation of pTK2 transfected cells as well as A-gene transformation of SV40 viral-DNA transfected cells (Fig. 2.12; A7, A8). TK-gene transformation appeared to be greater than A-gene transformation.



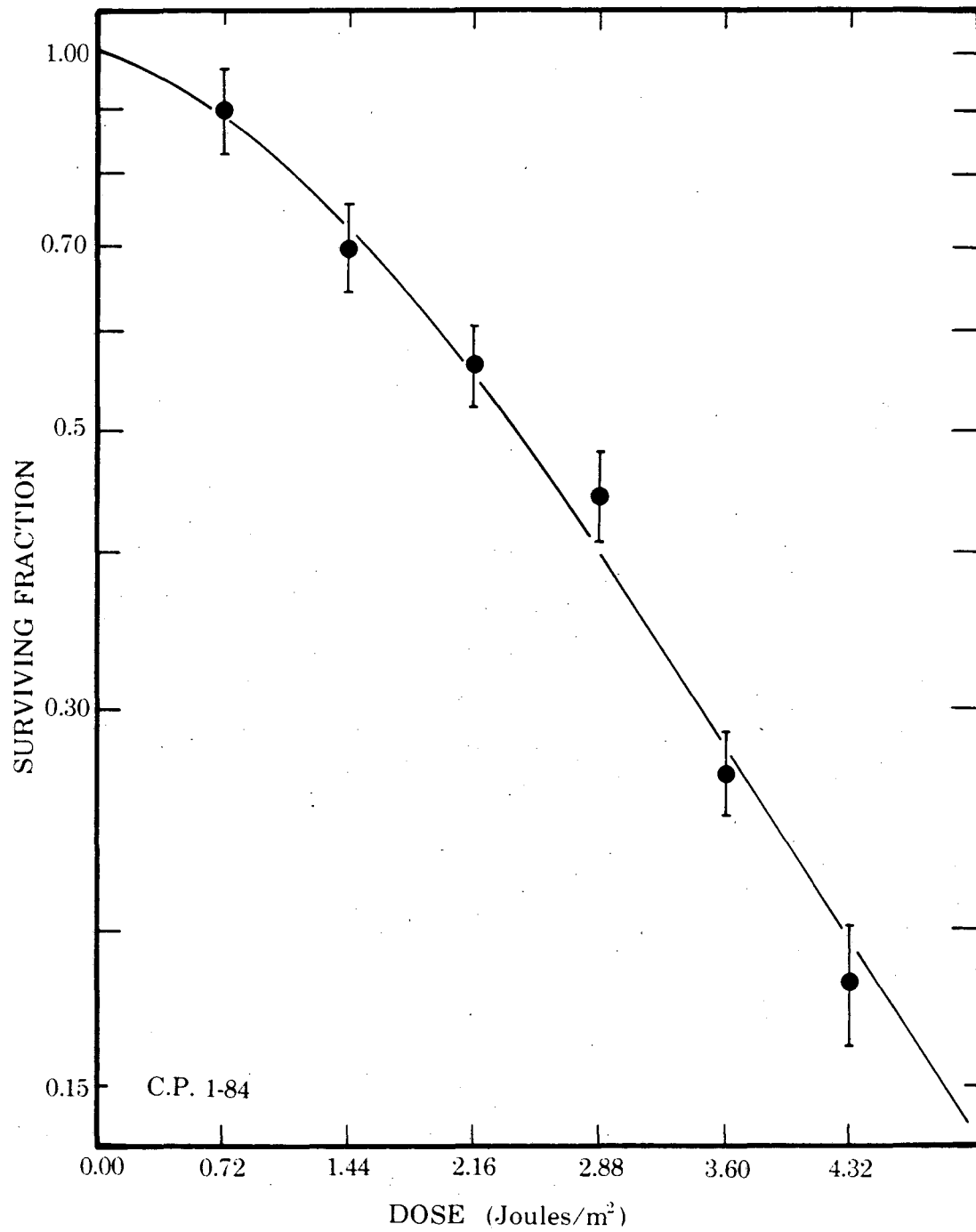
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Figure 2.9. X-ray enhancement of TK-transformation of pTK2 transfected Rat-2 cells.



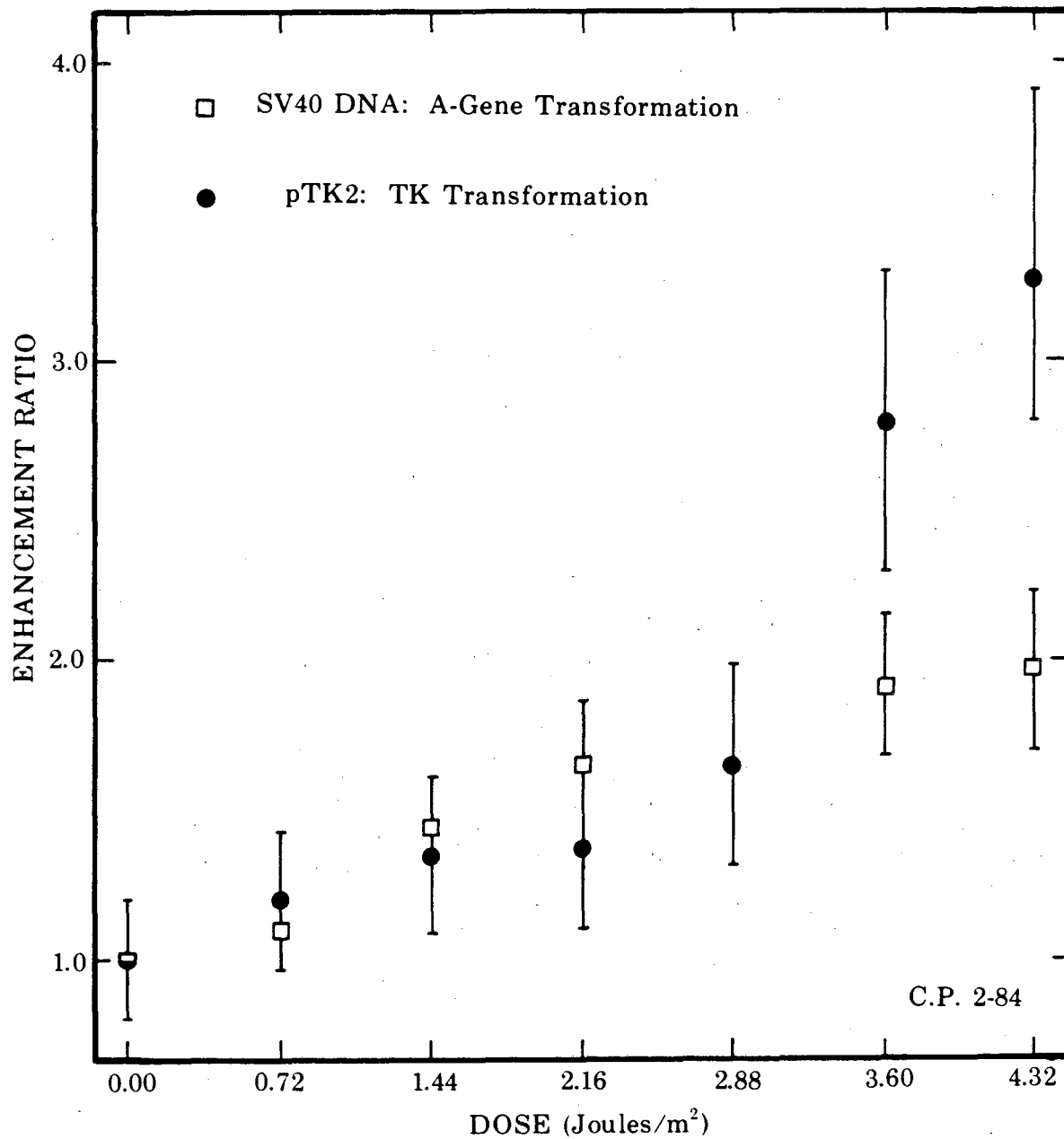
XBL 842-596

Figure 2.10. X-ray enhancements of A-gene transformation of SV40 DNA transfected Rat-2 cells and TK-transformation of pTK2 transfected Rat-2 cells.



XBL 842-598

Figure 2.11. Ultraviolet radiation survival curve of Rat-2 cells.



XBL 842-594

Figure 2.12. Ultraviolet radiation enhancements of A-gene transformation of SV40 DNA transfected Rat-2 cells and TK-transformation of pTK2 transfected Rat-2 cells.

What mechanism explains the enhancement of DNA mediated gene transfer by both ionizing and non-ionizing radiation? Presumably ionizing radiation produces DNA strand breaks, while ultraviolet radiation creates predominately pyrimidine cyclobutane dimers. DNA alkaline elution techniques have been used to study the formation of ultraviolet induced DNA single-strand breaks, which are produced by the action of the dimer specific endonuclease (Cleaver, 1974). This endonuclease may produce single strand breaks that are as recombinogenic as breaks produced by ionizing radiation.

One might ask whether lethal lesions are important for radiation enhancement. In Figure 2.13, I have plotted the enhancement ratios of TK-transformation for pTK2 transfected Rat-2 cells against the logarithm of the surviving fraction from both X-ray and UV experiments (A6, A7). We find an approximately linear relationship with no significant difference in slope between the X-ray and UV results. The apparent linear relationship between the enhancement ratio and the number of "lethal hits" is no proof that the lesions responsible for cell inactivation are also responsible for this effect (Munson and Goodhead, 1977).

Control experiments (data not shown) confirm that Rat-2 cells do not spontaneously revert to HAT⁺ phenotype (10^{-10}), nor do they revert upon irradiation. Since no morphologically transformed colony was found in irradiated non-transfected cell cultures during the short incubation time (21 days), the increase in foci was due to an enhancement of SV40 transformation. Similar work was performed by

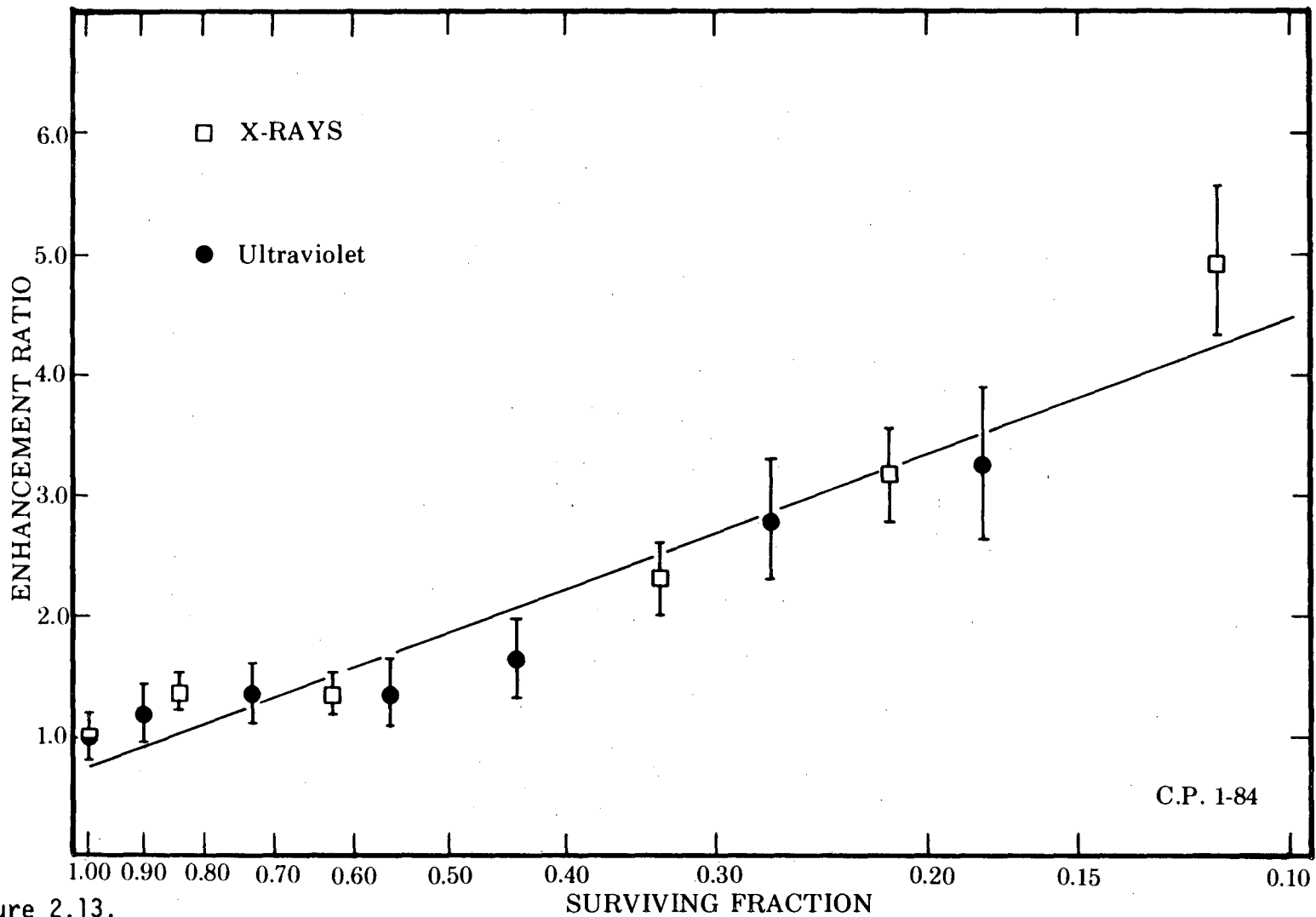


Figure 2.13. Enhancement ratios for UV and X-ray enhancements of TK-transformation of pTK2 transfected Rat-2 cells plotted as a function of the logarithm of the surviving fraction. XBL 842-597

Coggin (1969), who reported that of 50 SV40-transformed clones derived from preirradiated SV40-infected hamster embryo cells, 47 contained SV40-specific T antigen.

CONCLUSION

Ionizing and non-ionizing radiation enhances the efficiency of DNA mediated gene transfer. This phenomenon is observed with DNA of eukaryotic or prokaryotic origin and with DNA without any SV40 sequences. Since plasmid integration is necessary for the stable transformation of pTK2 transfected cells (Wigler et al., 1977; Huttner et al., 1981), increased integration of plasmid DNA during cellular radiation is implicated as the mechanism of radiation enhancement of DNA mediated gene transfer. Does radiation increase the amount of integrated DNA per individual cell, or does radiation treatment recruit more cells into a subpopulation that is more competent for genetic transformation? This question will be addressed in the next two chapters.

CHAPTER THREE

GENOMIC ANALYSIS OF ISOLATED TRANSFORMED CLONES

The working hypothesis suggested for the mechanism of the radiation enhancement of marker gene transformation was that transfected DNA is more efficiently integrated into the genome of an irradiated recipient cell. Does radiation increase the amount of DNA integrated per individual cell, or does it increase the efficiency of genetic transformation of the cell population?

This question may be addressed directly by examining the integrated DNA in transformed cell lines (Botchan et al., 1976). High-molecular weight DNA may be extracted from transformed cells and cleaved by sequence-specific restriction endonucleases. By using enzymes that do not cut the integrated plasmid sequences, we may get an estimate of the number of separate insertions in the mammalian genome, while multiple cutters give information on the amount of and the organization of the integrated DNA. The resulting DNA fragments are fractionated through an agarose gel, denatured in situ, transferred to a nitrocellulose filter (Southern, 1975), and hybridized to DNA that has been labeled to high specific activity by nick translation (Maniatis et al., 1975; Rigby et al., 1977). The distribution of the integrated sequences is determined by autoradiography. This technique is extremely sensitive, allowing the detection of as little as 0.1 pg of DNA in a band. By analyzing 15 μ g of cellular DNA, it is possible to recognize as few as 100 base pairs of foreign DNA per diploid equivalent of transformed cellular DNA.

Estimates of the organization of the integrated sequences can be made by selecting appropriate endonucleases. For this study pTK2 DNA was used to transform Rat-2 cells. From the physical map in the previous chapter (Fig. 2.2), we can select restriction endonucleases of three types. The first group consists of enzymes that are non-cutters of pTK2 (e.g., XBA I, KPN I). They give an estimate of the total number of pTK2 inserts in the genome, since the only fragments that can be generated are from cleavages in the flanking cellular sequences. By counting the number of bands that result from cleaving the transformed cellular DNA with these enzymes and hybridizing with nick-translated pTK2 DNA, we can obtain a minimum estimate of the number of separate insertions in the genome. However, each insertion may contain multiple copies of pTK2 (e.g. a tandem array). Enzymes of the second group (e.g., Sal I, Hind III, Bgl II) are single-cutters. They give information about the presence of integrated tandem copies or partially duplicated copies of pTK2. These enzymes will cleave within repetitious plasmid sequences and excise monomer-size copies of pTK2, which would migrate through agarose gels at the same rate as linear cut pTK2. And finally, the third class (e.g., Pvu II, Bam HI, Eco RI) will liberate internal fragments within the integrated plasmid sequences and can be used to estimate the number of integrated copies of pTK2. For this study, Xba I was used as the non-cutter, Hind III as the single cutter, and Pvu II as the multiple cutter of pTK2. Pvu II was selected because it frees the internal fragment that contains the TK gene. By serially

diluting Pvu II digested pTK2 DNA and comparing it to Pvu II digested transformed cellular DNA an estimate of the number of integrated pTK2 copies can be titered. By this technique the effects of ionizing radiation on the integration of plasmid pTK2 in transfected and transformed Rat-2 cells were studied.

MATERIALS AND METHODS

Transfections. Confluent Rat-2 cells were transfected in 100 mm dishes, in duplicate, with either 1, 10, or 50 $\mu\text{g/ml}$ of pTK2. After twelve hours, the media was changed with DME-10 percent FCS. After 24 hours, one set of plates were irradiated with 5.00 Grays of 225 kVp X-rays. All cells were trypsinized and plated into DME-10 FCS. 48 hours later, the media was changed with DME-10 FCS supplemented with HAT. Five clones from each of six conditions were established and expanded to mass culture for genomic DNA analysis.

Genomic DNA Analysis. Genomic DNA was prepared by a modification of the procedure of Graham et al. (1980) and is detailed in Appendix D.II. Fifteen micrograms of cellular DNA was digested overnight at 37°C with 45 units of the appropriate restriction endonuclease. The digested DNA was subsequently extracted with phenol, extracted once with ether, and precipitated with ethanol. The entire sample was electrophoresed in an agarose gel (0.8 percent or 1.0 percent, as noted), after which the gel was blotted by the method of Southern (1975) and the resulting nitrocellulose filter hybridized with nick translated DNA as noted. The complete details of the Southern transfer and hybridization are in Appendix G.

The 2.0 Kb Pvu II Tk-fragment of pTK2 was isolated by electro-elution. The slice of agarose containing the fragment was placed in a dialysis bag with electrophoresis buffer and electrophoresed at high voltage for 2 hrs. The polarity was reversed for 15 seconds, and the fragment was precipitated by ethanol from the eluent. The fragment was nick translated as before.

RESULTS

Figures 3.1 and 3.2 show the cleavage patterns of DNAs derived from thirty transformed cell lines of pTK2 transfected Rat-2 cells cut with Xba I. In both figures, lane a contains 15 pg of Sal I linearized pTK2 as a reconstruction control, which represents the expected amount of plasmid DNA that would be contained in 15 μ g of transformed cellular DNA if the pTK2 DNA were present as one single integrated copy per diploid equivalent. The arrow in both figures points to this 7.9 Kb band. Lane b contains 15 μ g of digested Rat-2 cellular DNA. As expected it contains no integrated sequences of pTK2. According to Figure 3.1, all the transformed cell lines from unirradiated cultures seem to contain one single insertion. The intensity of the bands compared to the reconstruction band suggests that the copy number is near unity, except for clone H50D, which appears to have a large amount of pTK2.

Figure 3.2 represents the Xba I cleavage patterns of cell lines from cultures irradiated with 5.00 Grays of X-rays. The cleavage pattern is not significantly different from that of the unirradiated

clones (Figure 3.1). There are clones with multiple insertions (HX10A, HX10B, HX50A), but the number of the bands are not significantly greater than unity.

Figures 3.3 and 3.4 show the cleavage patterns of the same transformed cell lines digested with Hind III. Clone H50D appears to contain repetitious DNA, evidenced by the intense band migrating at 7.9 Kb. Figure 3.4 shows the cleavage pattern of the irradiated cultures. Only two clones have any repetitious sequences, but there are extensive rearrangements of the integrated plasmid sequences.

Figure 3.5 depicts the Pvu II cleavage patterns of DNA extracted from transformed clones isolated from the unirradiated cultures shown in Panel A, and from the irradiated cultures represented in Panel B. To facilitate the determination of copy number, the nitrocellulose filters were probed with the nick translated 2.0 Kb Pvu II fragment of pTK2. Lanes a-c show the 2.0 KB band from Pvu II digested pTK2 of 75, 30, and 15 pg amounts, respectively. These quantities represent 5, 2, and one copies of integrated plasmid per cell, respectively. The unirradiated clones in Panel A show copy number of unity, except for clone H50D, which shows an approximate copy number of 4. Panel B demonstrates that X-ray irradiation produces a slight increase in copy number in the HX10 series, but not at levels significantly different from the unirradiated cultures that gave rise to the H10 series. It is clear that there is some rearrangement of plasmid sequences in transformed cells, as displayed by the additional bands.

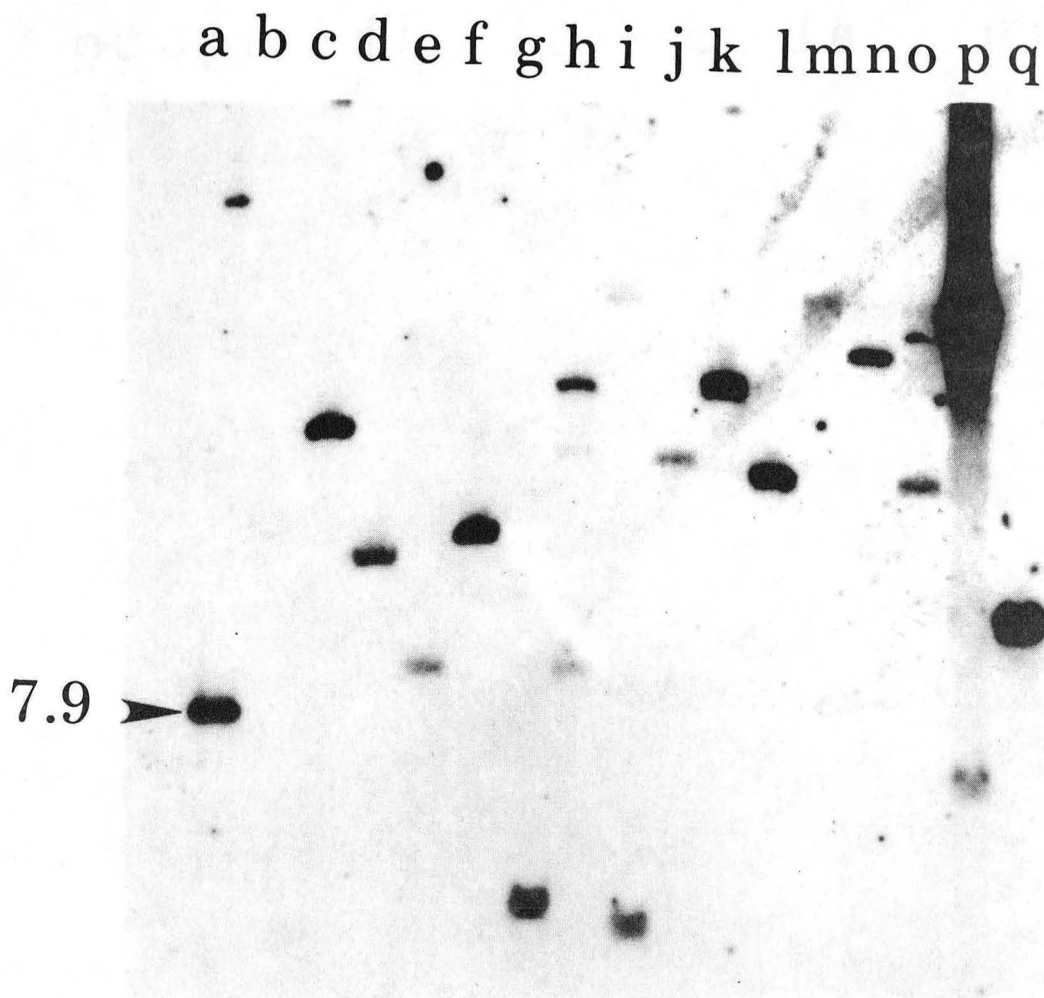


Figure 3.1. Xba I digests of high molecular weight DNA from HAT⁺ clones isolated from pTK2 transfected Rat-2 cells. Digests were fractionated on a 0.8% agarose gel, the filter was probed with ³²P-labeled pTK2 DNA, exposure was for 14 days.

lane a: 15 pg of Sal I digested pTK2 DNA
 lane b: Rat-2 DNA
 lanes c-g: clones H1A-E DNA
 lanes h-l: clones H10A-E DNA
 lanes m-q: clones H50A-E DNA

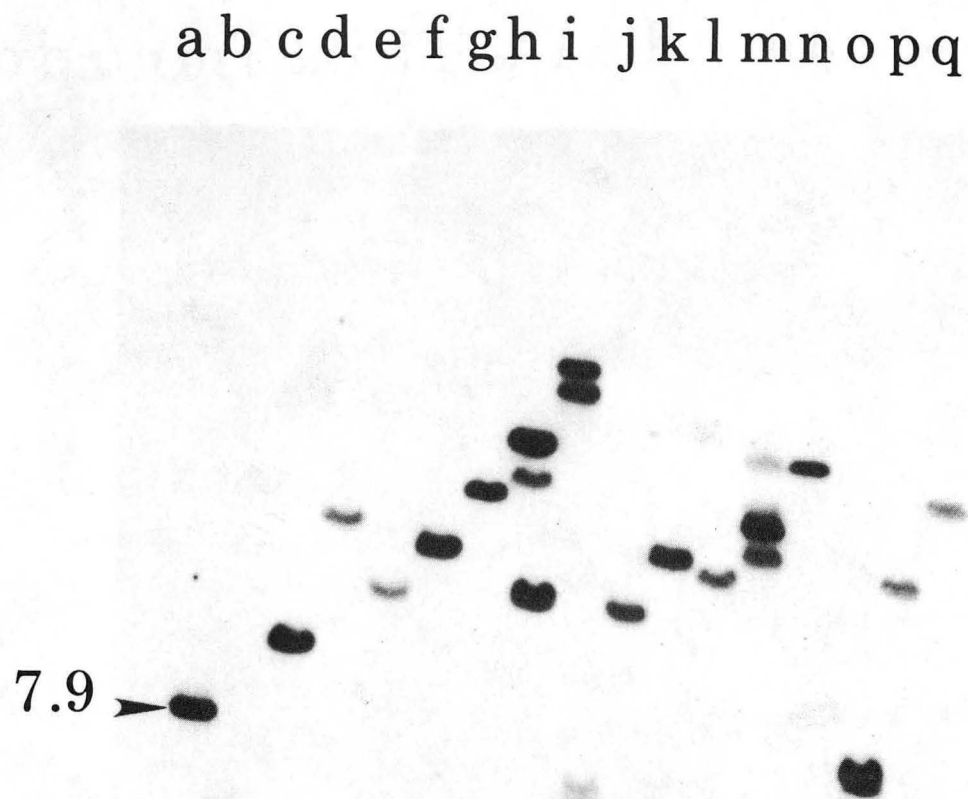


Figure 3.2. Xba I digests of high molecular weight DNA from HAT⁺ clones isolated from pTK2 transfected and X-ray irradiated (5.00 Grays) Rat-2 cells. Conditions described in Figure 3.1.

lane a: 15 pg of Sal I digested pTK2 DNA
 lane b: Rat-2 DNA
 lanes c-g: clones H1XA-E DNA
 lanes h-l: clones H10XA-E DNA
 lanes m-q: clones H50XA-E DNA

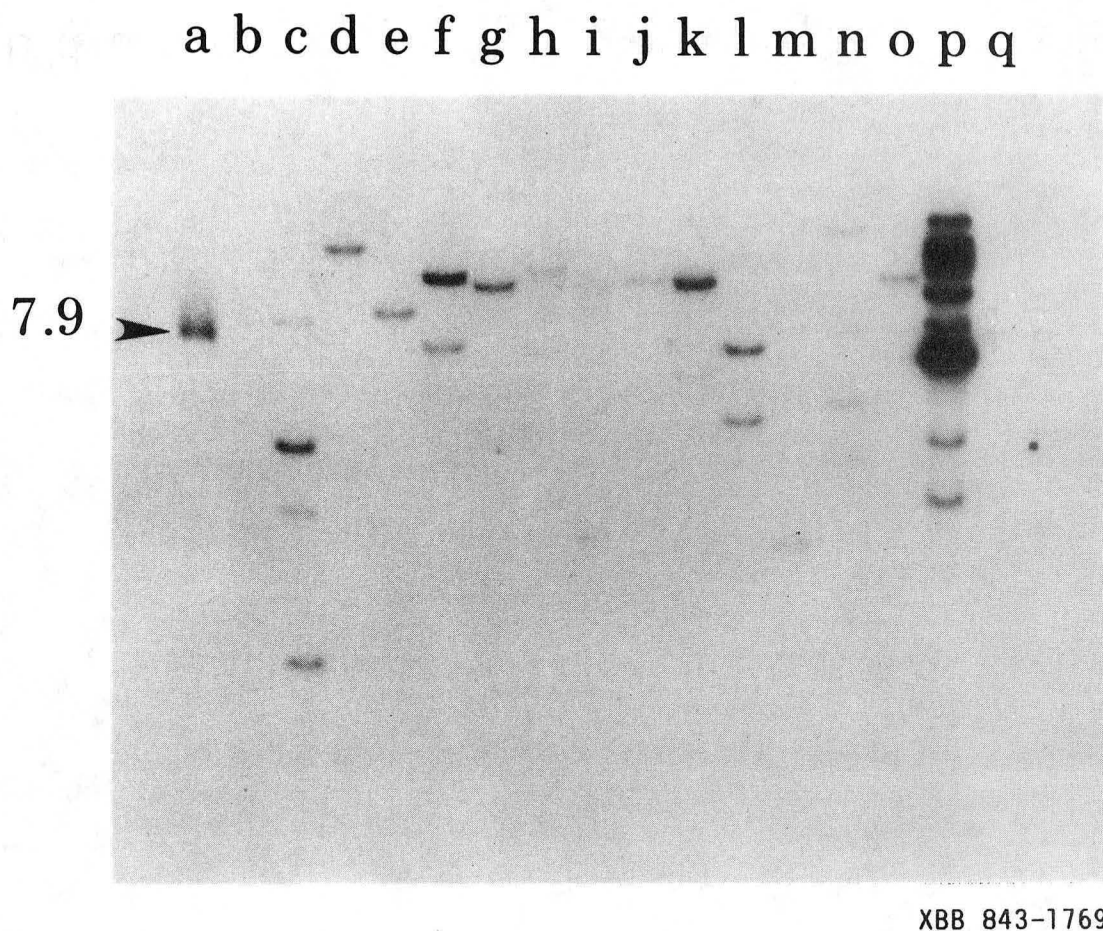


Figure 3.3. Hind III digests of high molecular weight DNA from HAT⁺ clones isolated from pTK2 transfected Rat-2 cells. Conditions described in Figure 3.1.

lane a: 15 pg of Hind III digested pTK2 DNA
lane b: Rat-2 DNA
lanes c-g: clones H1A-E DNA
lanes h-l: clones H10A-E DNA
lanes m-q: clones H50A-E DNA

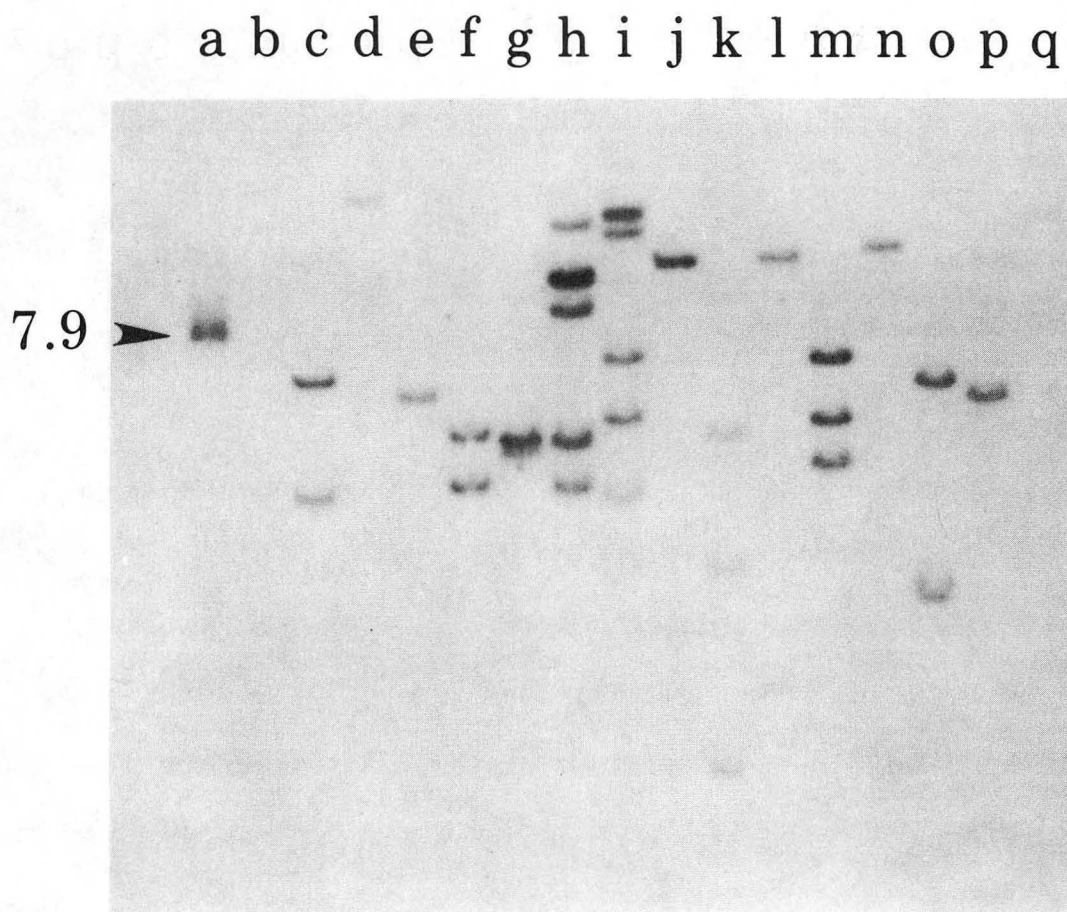
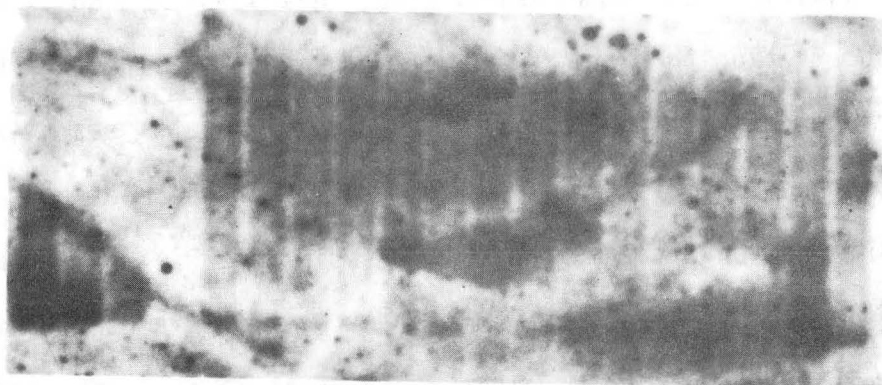


Figure 3.4. Hind III digests of high molecular weight DNA from HAT⁺ clones isolated from pTK2 transfected and X-ray irradiated (5.00 Grays) Rat-2 cells. Conditions described in Figure 3.1.

lane a: 15 pg of Hind III digested pTK2 DNA
 lane b: Rat-2 DNA
 lanes c-g: clones H1XA-E DNA
 lanes h-l: clones H10XA-E DNA
 lanes m-q: clones H50XA-E DNA

A a b c d e f g h i j k l m n o p q r s

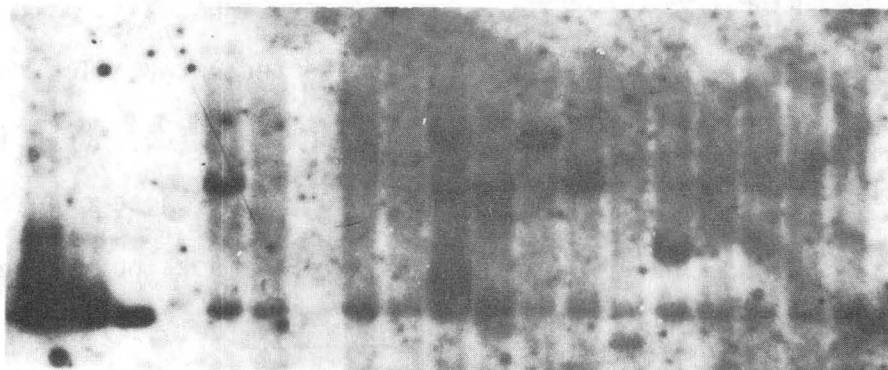
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lanes a-c: 75, 30, 15 pg of Pvu II digested pTK2 DNA
 lane d: Rat-2 DNA
 lanes e-i: clones H1A-E DNA
 lanes j-n: clones H10A-E DNA
 lanes o-s: clones H50A-E DNA

B a b c d e f g h i j k l m n o p q r s

2.0 ▶



lanes a-c: 75, 30, 15 pg of Pvu II digested pTK2 DNA
 lane d: Rat-2 DNA
 lanes e-i: clones H1XA-E DNA
 lanes j-n: clones H10XA-E DNA
 lanes o-s: clones H50XA-E DNA

Figure 3.5. Pvu II digests of high molecular weight DNA from HAT⁺ clones isolated from pTK2 transfected Rat-2 cells. Digests fractionated on 1.0% agarose gel, the filter probed with labeled 2.0 Kb TK-gene Pvu II fragment, exposed 14 days.

The different restriction fragment patterns generated in Figures 3.1 and 3.2 suggest that the plasmid, pTK2, integrates into the cellular genome at random sites. This point is supported by the observation that the flanking cellular XBA I sites are different distances from the plasmid insertion. The analyses from Figures 3.3, 3.4 and 3.5 infer that the plasmid itself does not possess a major integration attachment site. Plasmid integration is complex, as evidenced by the observation of many plasmid rearrangements—possibly a combination of non-homologous and homologous recombination (Wake et al., 1979, 1984; Miller and Temin, 1983; Gutai, 1981; Folger et al., 1982). The integration of pTK2 in pTK2 transfected Rat-2 cells appears similar to the integration of SV40 DNA in virally infected rodent cells (Botchan et al., 1976).

DISCUSSION AND CONCLUSION

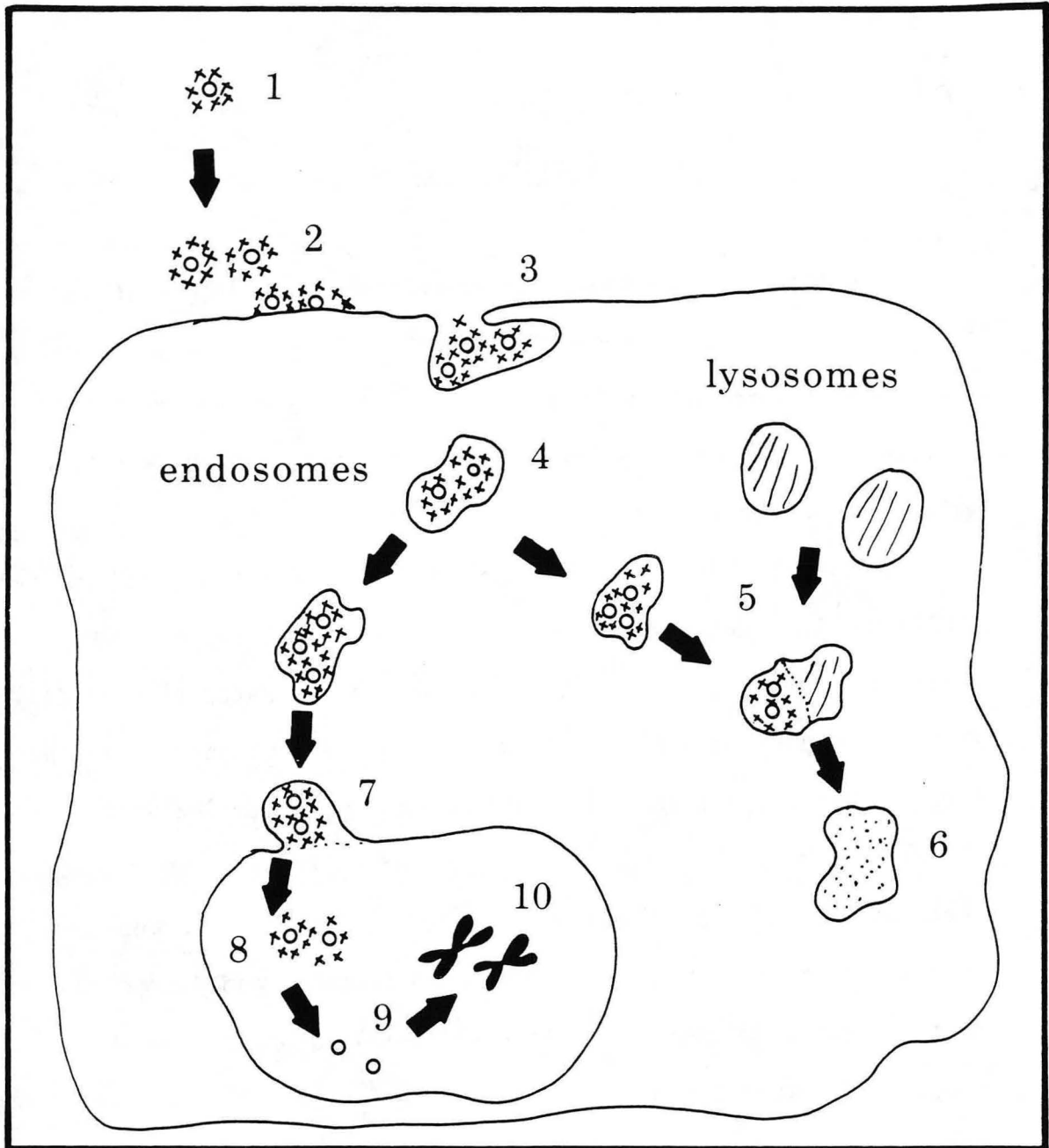
Despite the biological enhancement of approximately 8 times for 5.00 Grays of X-ray irradiated transfected Rat-2 cells, the amount of plasmid DNA integrated in clones established from these irradiated cultures is not significantly different from those clones established from control cultures. Ionizing radiation does not increase the copy number of transfected DNA in an individual cell. Ionizing radiation must increase the number of cells that are competent for genetic transformation. In the next chapter, we will explore this hypothesis further.

CHAPTER FOUR
MECHANISMS OF ENHANCEMENT

INTRODUCTION

I intended to study the physical effects of radiation on the competence of cells for gene transformation by two approaches: 1) DNA titration of marker DNA with and without radiation treatment, and 2) measuring the amount of DNA entering the cell nucleus with and without radiation treatment.

A thorough review of the mechanism of DNA mediated gene transfer (DMGT) has been written by Scangos and Ruddle (1981). A few key points will be considered here and will be illustrated schematically in Figure 4.1. The path of DNA-Ca precipitate through the cell has been visualized post-transfection by tagging the complex with fluorescent dyes (Loyter et al., 1982) and by in situ hybridizations (Lebkowski et al., 1983; Lusky and Botchan, 1981). Under optimum conditions of pH, temperature, DNA concentration, and DNA molecular weight, nearly 100 percent of the cells will adsorb the DNA-Ca precipitate (step 2 in Figure 4.1). After adsorption, 90 percent of cells may internalize the complex, presumably by pinocytosis (3), forming vesicles containing the DNA-Ca precipitate called endosomes (4). From a variety of observations, one may propose that the endosomes follow at least two pathways. Between 95 to 99 percent of the endosomes fuse with lysosomes (5), resulting in the degradation of the DNA (6). Alternatively, the endosomes surviving lysosomic fusion may instead fuse with the nuclear membrane (7) and in effect inject



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Figure 4.1. Schematic diagram for the proposed steps for DNA mediated gene transfer via calcium phosphate precipitation. See text for discussion.

the DNA-Ca precipitate into the nucleus (8). This scenario does not eliminate the possibility that partially digested DNA in the endosome-lysosome fusion vesicle will fuse with the nucleus, or that biologically active DNA reaches the nucleus by another pathway. After entry into the nucleus the exact mechanism of stable genetic transformation can only be surmised (9-10).

From the work of several laboratories (Lebkowski et al., 1983 and 1984; Calos et al., 1983; and others) it is clear that during transfection there is endonuclease attack, ligation of free ends, and nonhomologous and homologous recombination, resulting in the formation of a large mass of covalently linked DNA called a "transgenome." Indeed, elegant studies with engineered recombinant DNA plasmids demonstrate that transfection induces mutation in the input DNA at 1 to 5 percent frequencies (Lebkowski et al., 1983, 1984). These mutations consist of base substitutions, rearrangements, deletions, duplications, and insertions of host DNA. The transgenomes are inserted into the cellular chromosomes in a few random sites.

Several studies have demonstrated that transformed cell lines fall into two classes: those from which expression of the transferred gene is progressively lost (unstable cell lines) and those which retain gene expression even without selection pressure (stable cell lines) (Wigler et al., 1979; Graf et al., 1979; Lewis et al., 1980; Lester et al., 1980; Peterson and McBride, 1980). Cells that no longer express the gene physically lose the DNA.

In this study the radiation enhancement experiments did not employ carrier DNA in the transfection cocktail. The frequency of genetic transformation per microgram of plasmid DNA is two orders of magnitude greater with eukaryotic carrier DNA than without. But the frequency of stable cell lines per transformant is much higher with carrier-free transfection than with carrier. The functions that eukaryotic carrier may provide during DMGT are 1) DNA origins of replication, 2) signals for gene expression, and 3) a chromatin structure compatible for gene expression. Indeed, titration studies with different sources of carrier DNA show that procaryotic carrier DNA actually inhibits the efficiency of DNA mediated gene transfer (Yoder et al. 1983). Carrier-free transfections were performed in my studies to ensure that the transformants that arose by radiation enhancement were stable.

The ability of cells to uptake exogenous DNA and express the marker gene is defined as "competence." Only a subpopulation of cells within a culture is competent for genetic transformation by DNA mediated gene transfer. The competent phenotype is not stably inherited. Wigler and coworkers (1979) performed double transformations with murine cells that were double mutants, $Ltk^{-}aprt^{-}$. These cells were transformed first to either the $Ltk^{+}aprt^{-}$ or the $Ltk^{-}aprt^{+}$ phenotypes. Clones from the first transformation were transformed to the $Ltk^{+}aprt^{+}$ phenotype by a second transformation. Because there was no significant difference in the genetic transformation frequency of the first and second transformation, the researchers concluded that cell competence was a

transient property of the cells, possibly reflecting the metabolic state of the cells. An illustration of competence for genetic transformation can be found in gene marker titration, during which a population of cells is transfected with increasing amounts of marker DNA diluted with a constant amount of carrier DNA. The genetic transformation frequency rises linearly and then plateaus with increasing amounts of marker DNA. X-ray irradiation should proportionally enhance genetic transformation of this titration.

MATERIALS AND METHODS

High molecular weight DNA was isolated from LTK⁻ murine cells (Appendix I) as described in Appendix D.II. The DNA was aseptically sheared 10 times through a 22-gauge needle. Confluent Rat-2 cells were transfected in 100 mm dishes with 12 μ g of LTK⁻ carrier DNA mixed with increasing amounts of pTK2 DNA (45 to 900 nanograms). Duplicate cultures were transfected for 12 hours and then the media was changed to DME-10 percent FCS. After 2 additional hours, one set of dishes was irradiated with 5.00 Grays of X-rays. All cultures were trypsinized, counted, and plated for colony forming ability and for HAT selection as described before.

RESULTS AND DISCUSSION

Employing a constant large amount of eukaryotic carrier DNA, we see that the transformation frequency increases linearly with increasing concentration of plasmid pTK2 and saturates at about 5.3×10^{-4} transformants/survivor (Figure 4.2; A9).

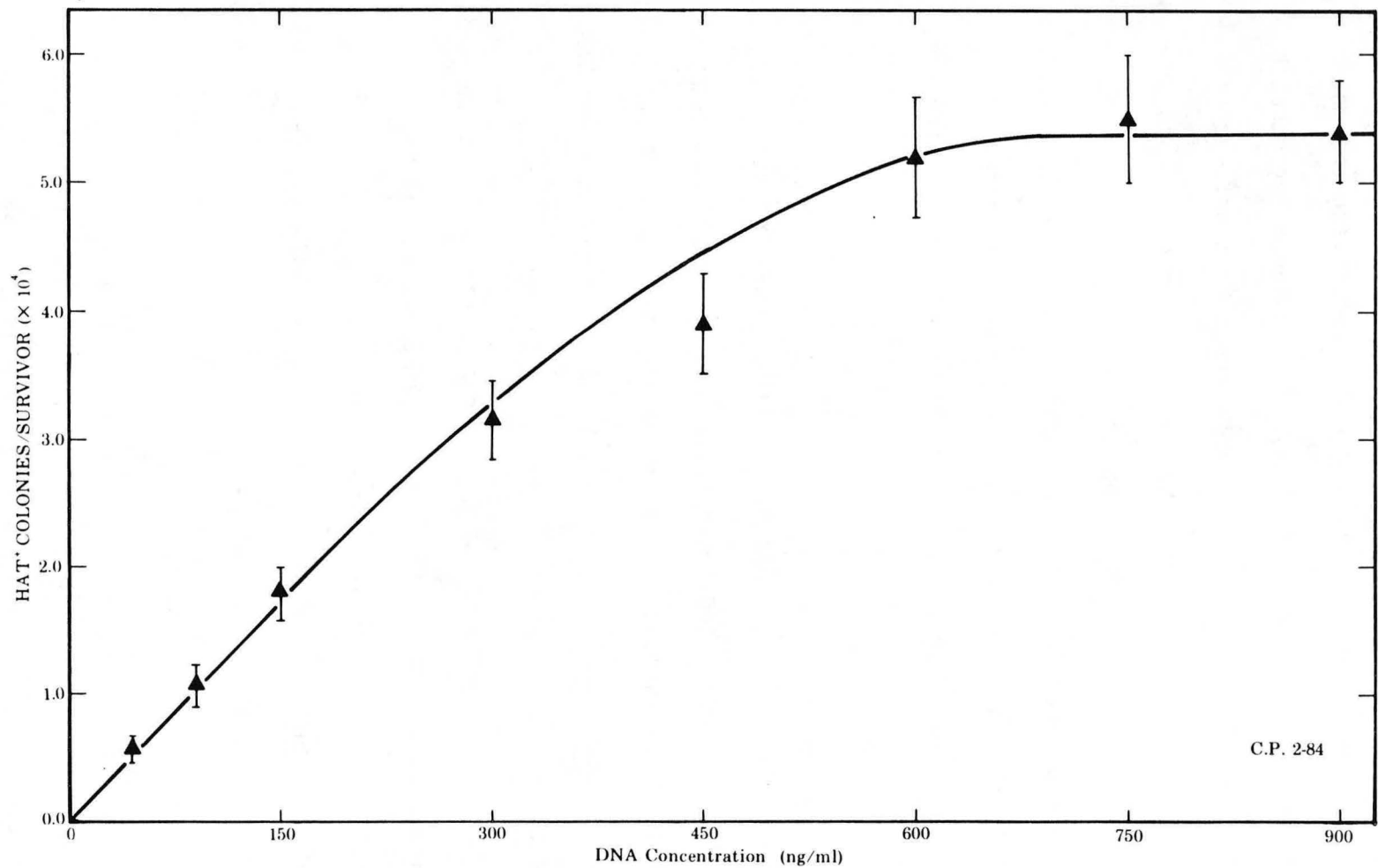


Figure 4.2. Plasmid pTK2 DNA titration of Rat-2 cells for TK-transformation, at constant carrier DNA concentration (12.0 micrograms/ml).

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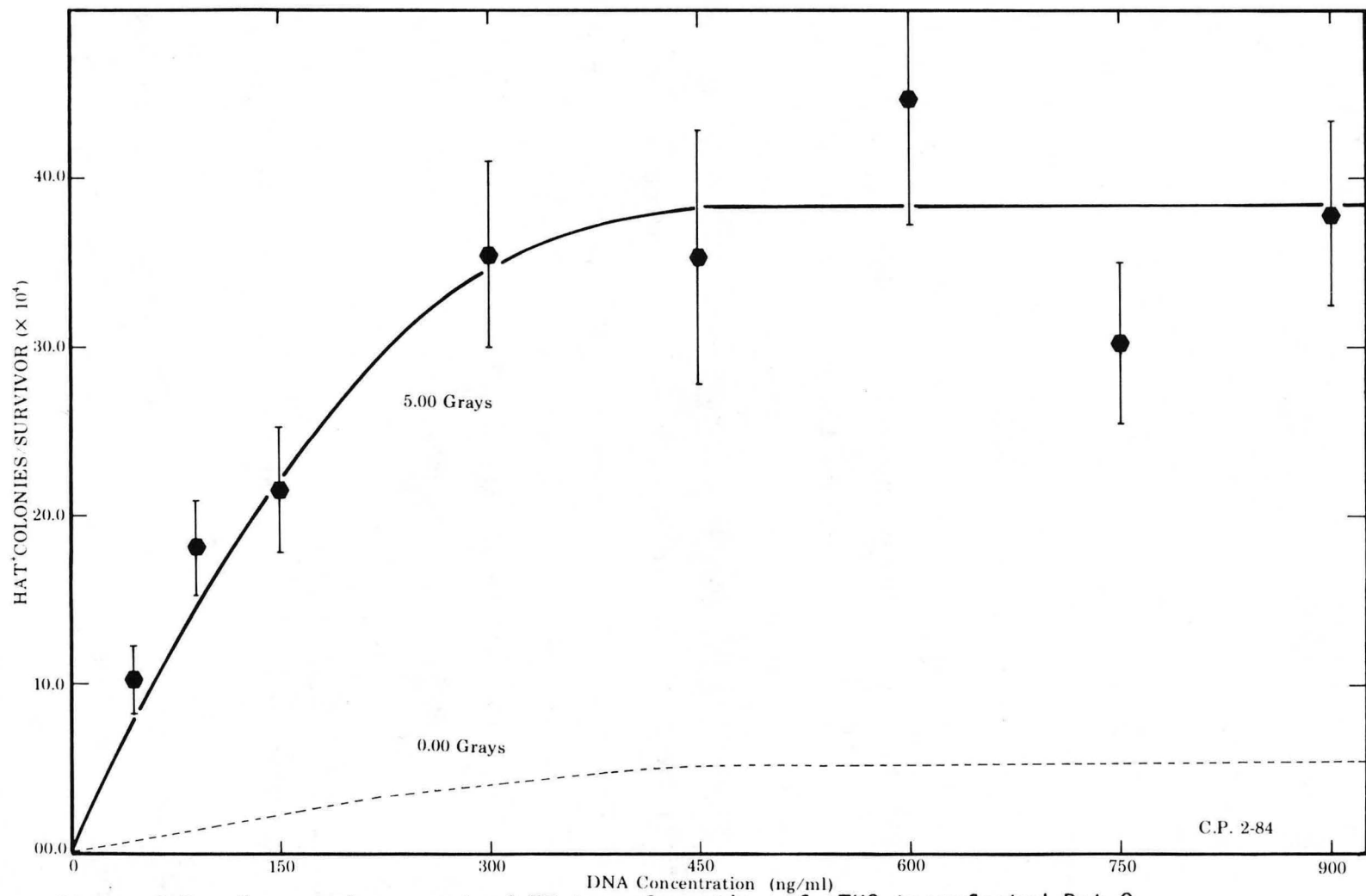


Figure 4.3. X-ray enhancement of TK-transformation of pTK2 transfected Rat-2 cells at constant carrier DNA concentration (12.0 micrograms/ml) and increasing concentration of plasmid pTK2 DNA.

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Upon irradiation with 5.00 Grays of X-rays, we see a concomitant enhancement of TK-transformation, which plateaus at about 38×10^{-4} transformants/survivor (Figure 4.3, A10). The 0.00 dose titration curve from Figure 4.2 is reproduced in Figure 4.3 for comparison. Radiation increases the competence of the population for genetic transformation.

All the experiments presented have relied on gene expression as the determinant of enhancement. To distinguish between increased nuclear uptake or increased integration as the mechanism of radiation enhancement of DNA mediated gene transfer, an experiment was designed to sensitively measure cellular and nuclear DNA uptake. Loyter and collaborators (1982) demonstrated that one could follow nuclear uptake with fluorescent dyes, but for statistical significance one would have to screen over 50,000 transfected cells at each dose to ascertain radiation enhancement of DMGT. Instead, I employed techniques already described in this study—DNA transfer to filters and DNA-DNA hybridizations—to tackle the enhancement question.

MATERIALS AND METHODS

16 100mm dishes of confluent Rat-2 cells were transfected with pTK2 DNA at a concentration of 14.4 $\mu\text{g}/\text{dish}$. After twelve hours the media was changed to DME-10 percent FCS. After an additional 24 hours, 8 dishes were irradiated with 5.00 Grays of X-rays. Three hours post-irradiation:

- A) Two unirradiated and two irradiated dishes were trypsinized, counted, and plated for colony forming ability and HAT selection for TK-transformation as described before.
- B) 5 plates of irradiated and 5 plates of unirradiated cells were rinsed twice with PBS and treated with 1 ml of lysis buffer B (0.12 M NaCl, 0.05 M Tris (pH 7.6), 0.5 percent Nonidet P-40). Lysis buffer B lyses the plasmid membrane and leaves the nuclear membrane intact, with the nuclei still attached to the tissue culture dish. The treated monolayers were rinsed gently with PBS to wash away cytoplasmic debris. The nuclei were quantitatively scraped into conical centrifuge tubes. The subsequent pellet was washed twice with PBS, and prepared for Hirt extraction (Appendix D.II.B.).
- C. One irradiated plate and one unirradiated plate were rinsed twice with PBS and prepared for Hirt extraction.

The lysates were collected in microfuge tubes, placed at -70°C for twenty minutes, and then spun to pellet out high-molecular weight DNA and protein. The supernatants were phenol extracted twice, ether extracted once, and precipitated with ethanol. The entire sample was electrophoresed through a 1.0 agarose gel. The gel was blotted as described before and the resulting filter was probed with nick translated pTK2 DNA. Each lane of the autoradiogram was quantitated by scanning with a LKB 2202 laser densitometer.

To calculate the number of copies of plasmid pTK2, C , by measuring the amount of DNA, D , in grams, we may use this formula:

$$C = DN_0/M.$$

Where D = amount of pTK2 DNA in grams,

M = molecular weight of pTK2 in grams/mole

N_0 = Avogadro's number, no. molecules/mole

The molecular weight pTK2 can be estimated from the relation;

$$M = BM_b,$$

Where B = number of nucleotide basepairs contained in the plasmid, 7960 bp.

M_b = average molecular weight of a nucleotide base pair, 600 grams/mole. Therefore, $M = BM_b = (7960) (600 \text{ grams/mole}) = 4.78 \times 10^6$ grams/mole.

The efficacy of the nuclei extraction was determined by pulse labeling two cultures of TK-transformed Rat-2 cell clone H1A (which contains one copy of pTK2) with 10 μ Ci of ^3H -thymidine for three hours and chasing for 10 hours. One culture was quantitatively extracted for nuclei as described above, the other culture scraped quantitatively for intact cells. Both samples were mixed with cold 5 percent perchloric acid and precipitated on Whatman glass fiber filters. The filters were washed extensively with cold 5 percent perchloric acid, cold 70 percent ethanol, 95 percent ethanol, air dried, and counted in a liquid scintillation counter in Aquasol.

Within a 95 percent confidence interval, the acid precipitable counts were the same from extracted nuclei as they were for total cells.

RESULTS

The autoradiogram (Figure 4.4) gives qualitative as well as quantitative information. The transfection process produced significant breakage, denoted by the large amount of form III DNA. The original plasmid preparation did not contain any observable form III, but it did contain pTK2 multimers. The arrow points to the form I species of the pTK2 dimer. X-ray irradiation eliminates the form I species (lane b) and also produces a smear of double stranded DNA fragments. The state of the DNA that actually exists in nuclei is depicted in lanes d and e. 24 hours post transfection we see form II- and form III-monomer and some form I-dimer. There are no qualitative differences of the DNA in the nuclei before and after 5.00 grays of X-rays. The results of the densitomer scans of this radiogram (App. K) are summarized in Table 4.1. Although there is no significant difference in the amount of DNA in the nuclei before or after irradiation, but there is a 15 percent more DNA in irradiated than the unirradiated cells. But from Table 4.2 we find that the biological enhancement ratio at 5.00 Grays is 9.23. While there is no significant increase in DNA in the nuclei, there is a biological enhancement of 923 percent. Enhancement of transformation occurs without a concomitant increase in nuclear uptake.

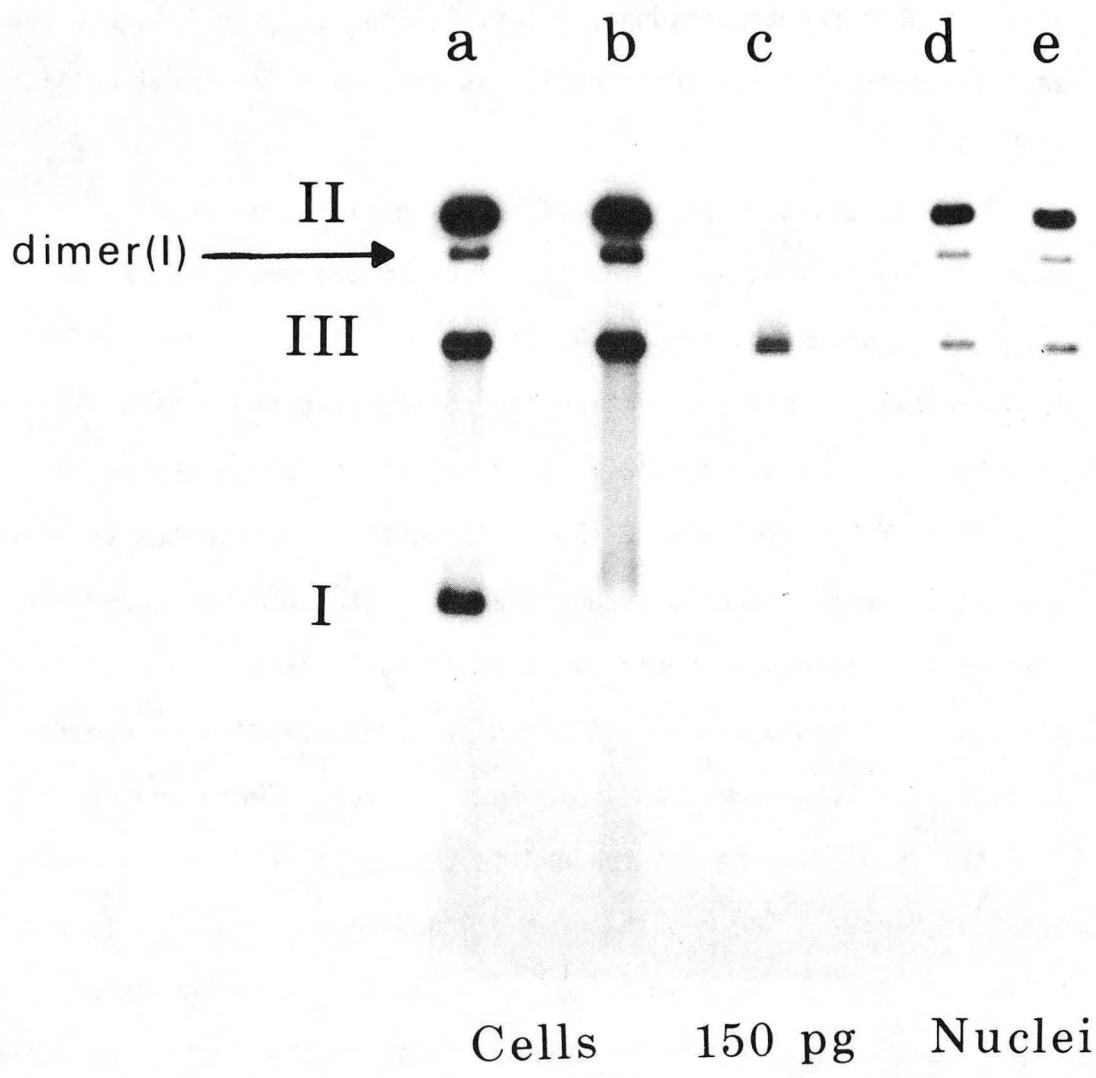


Figure 4.4. Hirt extracts of pTK2 transfected Rat-2 cells. The extracts were fractionated in a 1.0% agarose gel, the filter probed with ³²P-labeled pTK2 DNA and exposed for 10 hours.

- lane a: 2.25 x 10⁶ unirradiated cells
- lane b: 2.25 x 10⁶ irradiated (5.00 Grays) cells
- lane c: 150 pg of Sal I digested pTK2 DNA
- lane d: 22.5 x 10⁶ unirradiated nuclei
- lane e: 22.5 x 10⁶ irradiated (5.00 Grays) nuclei

Table 4.1.

Source of DNA	Amount DNA (pg)	Concentration of DNA	Average Copies of Ptk2
2.25 x 10 ⁶ unirradiated cells	1291	5.73 x 10 ⁻⁴ pg/cell	72/cell
2.25 x 10 ⁶ irradiated cells	1414	6.28 x 10 ⁻⁴ pg/cell	79/cell
22.5 x 10 ⁶ unirradiated nuclei	403	0.18 x 10 ⁻⁴ pg/nucleus	2.2/nucleus
22.5 x 10 ⁶ irradiated nuclei	409	0.18 x 10 ⁻⁴ pg/nucleus	2.3/nucleus
Reconstruction	150	-----	-----

Table 4.2. Radiation enhancement of TK-transformation of pTK2 transfected (14.4 µg/100mm dish) RAT-2 Cells

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish (x10 ⁻³)	Transformants Per Cell Plated (x10 ⁴)	Percentage Survival	Number of Transformants Per Survivor (x10 ⁴)	Enhancement Ratio
0.00	90.1 ± 4.9	304	2.96 ± 01.16	55.8 ± 3.8	5.3 ± 0.5	1.00 ± 0.12
5.00	59.2 ± 4.0	247	2.40 ± 0.16	4.9 ± 0.3	49.0 ± 5.2	9.23 ± 12.6

DISCUSSION

The previously described experiment gives information concerning the stoichiometry of DNA mediated gene transfer via calcium phosphate precipitation. Confluent monolayers (4.5×10^6) of Rat-2 cells were transfected for 12 hours, and then the media was changed. In the 24 hours before irradiation, the serum nucleases digested all the DNA precipitate on the cell surface (determined by microscopic examination of the cultures). Loyter and coworkers (1982) demonstrated that DNA rapidly degrades in MEM containing either heat treated or non-heat treated calf serum. Changing media after transfection removes most of the precipitate as well as the ionic environment (Hepes buffered pH 7.10, 0.012 M Ca^{++} , 0.014 M NaCl, 0.1 mM Na_2HPO_4) that maintains the precipitate. After media change, the DNA-ppt that has not been taken up into the cell dissolves in the media, after which the rapid degradation of DNA takes place. There can be no increased uptake of any significant amount of DNA into the cell at the time of irradiation. The efficiency of cellular intake as a population can be determined as follows:

1. The molecular weight of pTK2 is approximately 7.96×10^3 bp/mole
 $\times 600 \text{ g/bp} = 4.78 \times 10^6 \text{ g/mole}$.
2. The amount of DNA transfected per cell:
 $(14.4 \times 10^{-6} \text{ g}) \div (4.5 \times 10^6 \text{ cells}) = \underline{2.3 \times 10^{-12} \text{ g/cell}}$

3. We can convert this amount to copies of pTK2 per cell by

$$(3.2 \times 10^{-12} \text{g/cell}) \div (4.78 \times 10^6 \text{g/mole}) \times 6.02 \times 10^{23} \text{ copies/mole} =$$

$$\underline{4.03 \times 10^5 \text{ copies pTK2/cell transfected.}}$$
4. By similar calculations, the amount of pTK2 copies that enters the transfected cell (table 3.1) is 72 copies/cell.
5. Therefore, the average efficiency of cellular uptake is

$$(72 \text{ copies/cell}) \div (4.03 \times 10^5 \text{ copies transfected/cell}) = \underline{0.00018}$$

The efficiency of nuclear uptake relative to the amount of DNA that has actually entered the cell is:

$$(2.2 \text{ copies/nucleus}) \div (72 \text{ copies/cell};) = 0.031.$$

The value 3.1 percent agrees with the values found by observing nuclear uptake by fluorescent dyes bound to calcium-DNA precipitates (Loyter, et al., 1982) and in situ hybridization of SV40 tranfection (Calos et al., 1983).

But as stated before, mere entry into the nucleus does not assure biological genetic transformation. In fact, Luciw et al. (1983) microinjected pTK2 directly into the nuclei of mouse LTK⁻ cells. The microinjected cells were grown to mass culture and their genomes analyzed. Out of twenty clones that demonstrated integrated plasmid TK-gene sequences, only 3 (15 percent) expressed the tk-gene expression. Further studies by Folger and coworkers (1982) also showed that Rat-2 cells consistently show only 14 percent of the transformation frequency of mouse LTK⁻ by microinjection and DNA

mediated gene transfer. This finding suggests that in Rat-2 cells only $(0.15) \times (0.14) = .021 = 2.1$ percent of the cells that receive nuclear plasmid pTK2 DNA will show gene expression. From Table 4.2 we see that at 0.00 Grays (the control) the number of HAT⁺ transformants/cell plated is 2.96×10^{-4} . Or put in another way, the number of cells that need to be plated to give rise to one transformed clone is

$$1/(2.96 \times 10^{-4}) = 3.38 \times 10^3 \text{ cells plated/one transformant.}$$

Assuming the best recorded uptake of Ca-ppt (Loyter et al.) at 90 percent, we can approximate from this experiment the number of cells that have plasmid DNA in their nuclei:

$$4.5 \times 10^6 \text{ cells transfected} \times 0.90 \text{ uptake/transfected} \times \\ 0.031 \text{ nuclear uptake/cellular uptake} = 1.26 \times 10^5 \text{ cells} \\ \text{with nuclear plasmid DNA.}$$

The biological efficiency of cellular transformants per cell with nuclear plasmid DNA is:

$$(3.38 \times 10^3 \text{ cells/transformant}) \div (1.26 \times 10^5 \text{ cells with} \\ \text{nuclear DNA}) = 0.027 = 2.7 \text{ percent.}$$

This value agrees closely (2.7 percent vs. 2.1 percent) with that predicted from microinjected experiments. The previous mathematical exercise simply demonstrates that getting the DNA into the nucleus is not enough to assure biological transformation. While integration is one step that limits genetic transformation, stable expression of the integrated gene is also paramount. When nuclei of LTK⁻ are microinjected with plasmids that contain HSV-TK gene and a retroviral enhancer—long terminal repeat—85 percent of the cells that contain integrated sequences also express TK activity (Luciw et al., 1983; Folger et al., 1982). Radiation may serve to increase the integration of plasmid DNA into a population of cells, which concomitantly increases the probability that a TK-gene will integrate into a region or domain of cellular DNA that is compatible for gene expression.

Radiation enhances the efficiency of DNA mediated gene transfer via calcium phosphate precipitation. Though radiation does not significantly increase the amount of integrated DNA per individual recipient cell, it does increase the number of cells in a population that are competent for genetic transformation. This increased competence is not due to increased nuclear uptake. There are three models that can explain these results: 1) targeted integration, 2) partitioning of cellular domains, and 3) inducible recombination.

Targeted integration model. This model states that radiation increases the production of DNA strand breaks, which are substrates for DNA integration. X-rays produce predominantly single strand

breaks, but even argon particles with an LET of 144 KeV/micron still produce a reasonable amount of single strand breaks. Ultraviolet enhancement occurs as a consequence of the repair of pyrimidine cyclobutane dimers. The dimer specific endonuclease produces single strand breaks that may be used as integration sites.

There will be an increase in the number of random insertions with increasing radiation dose. But since the transformation frequencies are extremely low, the probability of seeing more than one copy per transformant is also very low. Probability calculations can be made concerning the number of integrated copies/transformant. Using the Poisson distribution, the expected probability of the number of separate DNA insertions per transfected cell is calculated by

$$P(x) = m^x e^{-m} / x!$$

where x is the number of separate insertions per transfected cell, and m is the average number of transformants per transfected cell. This calculation implies that integrated DNA will give rise to transformants. From the data in Table 4.2, we see the average number of transformants per transfected cell for unirradiated cells is $m = 5.3 \times 10^{-4}$, and for irradiated cells is 49.0×10^{-4} . The probabilities are calculated below:

	UNIRRADIATED	IRRADIATED
P(0)	1.00	1.00
P(1)	0.0005	0.005
P(2)	0.0000001	0.00001
P(3)	2.5×10^{-11}	2.0×10^{-8}

In both cases the probability of finding multiple insertions is extremely small. Since genomic analysis was performed only on transformants, we find that even in irradiated cells there would be approximately one transformant with more than one insert for every 500 transformants (0.005/0.0001).

The genomic analyses may argue against a simple target model due to the observation of multiple insertions in both irradiated and unirradiated clones. In clone H50D we see a large amount of DNA integrated at two sites. Rearrangements are seen in all of the multiple inserted integrated DNAs. The Xba I digests point to multiple insertions in the irradiated clones (H10XA, H10XB, H50XA) and unirradiated clones (H1D, H10E, H50D). During the complex integration process cellular DNA containing Xba I recognition sequences may have rearranged with the plasmid DNA. Multiple inserts shown by Xba I digests of transformed cellular DNA may actually be explained by complex rearrangements and not multiple integration events within the transformant. This possibility may be tested by performing in situ

hybridizations of metaphase chromosome preparations on microscope slides and screening the chromosomes of the transformed clones for multiple insertions.

The targeted integration model based on the observations reported by Yang et al. (1980) is still attractive. When SV40 infected rodent cells were irradiated in confluence and plated at different time periods post-irradiation, the enhancement ratio for SV40 transformation decreases. This implies that damage to the chromosomes is repaired before the cells are committed to division--leaving less substrates for integration.

One caveat must be mentioned before making strict parallels between SV40 transformation via viral infection and DNA transfection. After SV40 viral infection, the virus particle enters the nucleus, where it is uncoated, leaving the viral DNA still complexed with the nucleosomes originating from the monkey kidney cell that supported its production. Loyter and coworkers (1982) showed that the DNA-calcium precipitate enters the nucleus intact. It is not known where or when the transfected DNA complexes with the recipient cell's nucleosomes. I have confirmed (data not shown) what others (Botchan et al., personal communication) have reported about SV40 viral infection possibly being quite different than SV40 viral DNA transfection. If rodent cells are infected with a temperature sensitive A-gene virus (tsA-209; Chou and Martin, 1974) at the non-permissive temperature and then shifted to the permissive temperature, no transformants are recovered. Yet if purified tsA-209 DNA is transfected into rodent

cells at the non-permissive temperature and then shifted to the permissive temperature, transformants are recovered. The production of viable T-antigens may be necessary for the integration of a SV40 mini-chromosome (with monkey cell nucleosomes) but may not be necessary for naked SV40 DNA. Alternatively the recipient cell's nucleosomes may increase DNA integration.

The targeted integration model may be tested by using cell lines such as Xeroderma pigmentosum that are incapable of performing the first step in the repair of UV pyrimidine dimers—the dimer specific endonuclease step. Busch and coworkers (1980) have reported the isolation of mutant CHO cells that are UV sensitive and complement the various Xeroderma pigmentosum complementation groups. CHO cells are efficient for DNA mediated gene transfer and have high plating efficiencies. By transfecting the neomycin dominant selectable marker (Colbere-Garapin et al., 1981) into a UV-sensitive CHO mutant that complements XP group A, the targeted integration model predicts that X-rays but not UV irradiation will enhance DNA mediated gene transfer. This prediction is based on experiments that show XP group-A cells repair X-ray damage as normal cells, but cannot produce nicks in DNA after UV irradiation (Fornance et al., 1976).

Partition Model. This model states that radiation does not increase the probability of integration of DNA per cell, but changes the ability of the cell to express the integrated DNA. The mammalian genome consists of actively expressed and dormant domains of

chromatin. DNA that integrates in a non-active cellular domain will not be expressed unless the domain is "activated" by irradiation. This model fails to explain one major experimental observation. From work performed in collaboration with Michael Kriegler and Michael Botchan (Kriegler et al., 1983; and manuscript in preparation), we and others (Khoury and Gruss, for review, 1983) have shown that enhancers increase gene expression and not the amount of DNA integrated in a transformant. Depending on the cell-specific enhancer (Kriegler et al., 1983), an enhancement of greater than one order of magnitude in DNA mediated gene transfer is observed. The recombinant-plasmid, pOT-TK5, contains the SV40 enhancer sequence. The partition model predicts a decreased X-ray enhancement of TK-transformation for pOT-TK5 compared to pTK2 transfected Rat-2 cells. Thymidine kinase genes that would be expressed during radiation activation of their cellular domain would have already been activated by their enhancer sequences. But the experimental observations (Appendix Tables A5 and A6) show that enhancement does occur at the same order of magnitude as pTK2 transfected Rat-2 cells.

Inducible recombination model. Radiation may serve to induce the expression of recombination genes that increase the frequency of DNA integration. This model may be called the "untargeted" integration model, since integration of the transfected DNA would occur at sites that are not radiation induced lesions.

There are experimental observations of induced recombination. Rodent cells are transformed by SV40 virus presumably because they do

not possess the "factors" necessary to complement T-antigen mediated viral replication. The COS-7 cell line was constructed by transfecting CV-1 cells with a SV40-plasmid that was mutated at the origin of replication (Gluzman, 1981). The COS-7 cell line is morphologically transformed, produces normal T-antigens, and even supports the replication of SV40-recombinant plasmids that contain only the SV40 origin (Lusky and Botchan, 1981). An intact SV40 origin is therefore needed for replication. The SV40-transformed Rat-1 cell line, on the other hand, contains an intact SV40 early region and intact origin of replication. It is also morphologically transformed and makes normal T-antigens, yet it does not produce any replicating DNA. When permissive CV-1 cells are fused with Rat-1 cells, SV40 sequences are excised in the heterokaryon. CV-1 cells produce the permissive factors that allow for SV40 replication and excision lacking in transformed cells. An enhanced amount of SV40 excision products is observed if the CV-1 cells are pretreated with the DNA damaging agent Mytomycin C before fusion (Miller, Bullock and Botchan, manuscript submitted). This result suggests that Mitomycin C induces trans acting factors that enhance SV40 excision—a DNA replication and recombination process.

Lambert and collaborators (1983) have shown that damage to DNA induces enzymes involved in polyoma viral excision. They found that the amount of polyoma excision products could be enhanced by fusing normal rat fibroblasts pretreated with BPDE (benzo-a-pyrene trans-7, 8-dihydrodiol-9, 10-epoxide(anti)) to unexposed polyoma transformed

rat fibroblasts. This result demonstrated that DNA damage to nonpolyoma-containing cells induces trans acting cellular factor(s) that stimulate polyoma excision upon fusion. It would appear that non-permissive rodent cells do contain the factors necessary to promote papovavirus DNA synthesis and recombination if they are induced by an appropriate DNA damaging agent. Lavi and Etkin (1981) have shown that low levels of carcinogens can induce the synthesis of SV40 DNA in SV40 transformed Chinese hamster embryo cells. These findings are in accord with the "onion-skin" model of excision of SV40 DNA from transformed cells (Botchan et al., 1978). Heterokaryon fusions with either permission CV-1 cells or activated rodent cells, as well as transformed cells treated with DNA damaging agents, produce the factors necessary to complement T-antigen mediated replication of the integrated SV40 origin. The polytenized SV40 DNA sequences that result from the in situ replication of the SV40 origin are now substrates for recombination enzymes—induced or constitutive—that complete the excision process. Since SV40 excision requires such an intimate coordination of replication and recombination, it is not clear that recombination genes are constitutively expressed or induced by DNA damage. DNA replication enzymes may not be necessary for DNA integration, suggesting that DNA damage may induce DNA synthesis genes but not the genes needed for DNA recombination. Indeed, the transfection process is not simple. Newly transfected DNA is subjected to many mutagenic lesions, including deletions, inversions, and translocations (Lebkowski et al., 1984; Calos et al., 1983). This suggests that newly transfected DNA is by itself recombinagenic.

Spivak and coworkers (1984) reported enhanced transformation of human cells by UV irradiated plasmids. They also stated that the increased frequency of transformation required the irradiation of the DNA itself and was not an indirect effect of the presence of damaged DNA in the recipient cell. Unirradiated pSV2-gpt plasmid DNA transfected together with UV-irradiated pSV2-neo plasmid DNA produced no more transformants than when it was introduced with unirradiated pSV2-neo. Yet irradiation of the pSV2-neo increased the yield of neomycin resistant transformants. These workers proposed two hypotheses to explain their findings. Cellular processing of damaged plasmid DNA might enhance DNA recombination and hence integration. The other hypothesis, reminiscent of the partitioning model, suggested that UV induced alterations of the plasmid DNA may facilitate the expression of integrated sequences. This second hypothesis may not be tenable due to the fact that both plasmids contain the SV40 enhancer sequence that in and of itself increases gene expression.

CONCLUSION

In toto, the mechanism of the radiation enhancement of DNA mediated gene transfer may involve "targeted" integration of plasmid DNA at the increased number of strand breaks induced by irradiating cells. Alternatively, the mechanism may involve "untargeted" integration produced by inducible DNA recombination enzymes. Or more likely, a combination of both models may ultimately be true. The question of the existence of inducible DNA repair in mammalian cells is still unanswered.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

We believe that this dissertation is the first work that describes the enhancement of DNA mediate gene transfer by a physical process. This phenomenon was studied with three different modalities of radiation: 1) 225 KVp X-rays; 2) 330 MeV/amu argon ions, and 3) ultraviolet.

This enhancement phenomenon appears to a general one. Two gene markers were studied--the SV40 A-gene and the Herpes Simplex Virus thymidine kinase gene--and in both cases the kinetics of enhancement were the same for either marker gene in cells treated with the same modality of radiation. Indeed, cells transfected with a recombinant DNA plasmid that contained both of these genes demonstrated identical enhancement with X-rays. The transfected DNA may be of either procaryotic or eucaryotic origin to produce the same enhancement.

The enhancement mechanism was studied in two ways. The first approach was to analyze 30 individual clones of cells for the amount of and organization of the integrated plasmid DNA in their genomes. The second approach was to quantitate the amount of plasmid DNA that entered the cytoplasm and the nucleus after the transfection process with and without radiation treatment. Radiation did not increase the amount of plasmid DNA that entered the cell nor into the nucleus, nor did it increase the amount of integrated DNA per individual recipient cell. We concluded that radiation must increase the competence of the cell population for genetic transformation by recruiting more cells

into a subpopulation of cells that are more able to integrate the very low amount of plasmid DNA that enters the cellular nucleus. The physical limitations of the transfection process made it impossible to perform transfection experiments with saturating amount of plasmid DNA.

The closest analogue to this work is that of the radiation enhancement of SV40 A-gene transformation via viral infection. The radiation enhancement of SV40 A-gene transformation via viral infection was similar to that seen via DNA mediated gene transfer. But, as stated earlier, there may be a very big difference in the mechanism of integration when the foreign DNA carries its own nucleosomes (as in SV40 viral infection) versus when the DNA enters the nuclei without complexed histones. It can be speculated that a rate limiting step in DNA recombination may be in that the nucleosomes must be extricated from the DNA to expose the chromosomes for DNA recombination and integration.

Nevertheless, the SV40 A-gene does not contribute to the radiation enhancement of DNA mediated gene transfer. Any marker gene that allows for an expressible phenotype can be used for radiation recombination studies in the future.

Ionizing and ultraviolet radiation has now been shown to enhance the efficiency of genetic transformation by DNA mediated gene transfer. This radiation enhancement is a general cellular phenomenon not limited to SV40 viral infection nor any SV40 A-gene functions. It is not clear whether integration occurs at the site of DNA damage—targeted integration—or whether in the process of repairing

DNA damage the exogenously introduced DNA integrates at secondary sites—untargeted integration. This study did not prove or disprove the existence of inducible repair in mammalian cells. Due to the large size of the mammalian genome, it may be more efficient to maintain a constitutive level of enzymes to continually relax and ligate DNA that is undergoing local supercoiling due to transcription, replication, and condensation. Radiation may produce DNA damage that is repaired by the constitutive apparatus.

The techniques presented in this study may be employed as an assay for "misrepair" or increased DNA recombination as the result of irradiated transfected cells. This approach offers these advantages:

1. Studies of DNA recombination can now be extended to mammalian cells and not limited to lower eukaryotes or prokaryotes.
2. The DNA that serves as the "vector" of integration is well characterized by its primary structure and biological function.
3. It is not necessary to use large amounts of radioactively labeled DNA precursors—which may perturb normal DNA metabolism.
4. Established clones from recombination events may be isolated and analyzed for their integrated DNA via gel blotting and hybridizations.
5. The techniques are reproducible and quantitative, allowing for kinetic studies of DNA recombination in mammalian cells.

DIRECTIONS FOR FUTURE RESEARCH

The application of DNA mediated gene transfer creates new opportunities for radiation biology. All established approaches in

radiation biology--split dose, dose modifiers, different modalities of radiation, radiation sensitive mutants, etc.--can be combined with gene transfer techniques to study DNA recombination.

This study involved biochemical transformation of a thymidine kinase gene into thymidine kinase deficient rodent cell line--Rat-2. This marker system poses a limitation for those using well characterized cell lines that are not thymidine kinase deficient. But there now exist some very well characterized dominant selectable gene markers that may be used with any mammalian cell line. The bacterial neomycin gene can be modified so that it will be expressed in mammalian cells (Colbere-Garapin, et al., 1981). This "eukaryotic neomycin" resistance gene confers G418 drug resistance to mammalian cells. The bacterial gpt gene that codes for xanthine-guanine phosphoribosyl transferase can be similarly modified to confer resistance to cells plated in media containing mycophenolic acid and Xanthine (Mulligan and Berg, 1980, 1981). The mouse dihydrofolate resistance gene has been used successfully to confer methotrexate resistance (Subramani et al., 1981). Many cell lines have been established that are sensitive to ultraviolet radiation (Busch et al., 1980; Thompson et al., 1980, 1981) and that have been shown by cell fusion to complement human Xeroderma pigmentosum cell lines. These rodent lines have high plating efficiencies and are efficient for DNA mediated gene transfer. Radiation enhancement of dominant selectable marker transformation of mutant cell lines could shed a different light on the nature of the actual defect leading to radiation sensitivity.

The actions of various drugs in conjunction with radiation can also be studied by DNA recombination. By DMGT and mutant cell lines we could study the effects of various inhibitors of DNA polymerases (aphidicolin, 1- β -D-arabinofuranosyl, dideoxythymidine), protein synthesis (cyclohexamide), topoisomerases (nalidixic acid), poly(ADP-ribose) synthesis (3-aminobenzamide), as well as dose modifiers (misonidazole, SR-2508). By characterizing pulse-transfection techniques (30 minutes or less), cell synchrony experiments with automated mitotic shakers can assess the cellular age response of DNA recombination and concomitant radiation enhancement. This study also demonstrated the efficacy of transfection with confluent monolayers of cells that are density dependent contact inhibited. Sub-lethal and potentially-lethal damage repair and their relationship with DNA recombination may be investigated.

. DNA mediated gene transfer offers ample opportunity to study a new endpoint for radiation repair—DNA recombination—in mammalian cells using dominant selectable gene markers in a reproducible and quantitative way.

Table A1. X-ray Enhancement of A-Gene Transformation of SV40 Viral DNA Transfected (12.5 μ g/100 mm dish) RAT-2 Cells

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	132.4 \pm 10.3	223	5.94 \pm 0.46	75.4 \pm 4.2	7.9 \pm 0.8	1.00 \pm 0.14
1.00	182.4 \pm 12.1	215	8.48 \pm 0.56	64.9 \pm 4.0	13.1 \pm 1.2	1.66 \pm 0.22
2.00	166.8 \pm 11.6	247	6.75 \pm 0.47	50.0 \pm 3.8	13.5 \pm 1.4	1.72 \pm 0.24
3.00	120.4 \pm 9.8	217	5.55 \pm 0.45	36.0 \pm 3.0	15.4 \pm 1.8	1.96 \pm 0.30
4.00	122.0 \pm 9.9	229	5.33 \pm 0.43	15.2 \pm 1.3	35.1 \pm 4.2	4.45 \pm 0.68
5.00	64.2 \pm 7.2	205	3.13 \pm 0.35	8.7 \pm 1.1	36.0 \pm 6.0	4.57 \pm 0.88
6.00	79.6 \pm 7.9	437	1.82 \pm 0.18	2.0 \pm 0.2	91.1 \pm 13.5	11.57 \pm 2.05

Table A2. X-rays Enhancement of TK-Transformation of POT-TK5 Transfected (1.71 ug/100mm dish) RAT-2 cells.

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	84.6 \pm 6.9	191	4.43 \pm 0.36	39.4 \pm 2.6	11.3 \pm 1.2	1.00 \pm 0.15
1.00	121.1 \pm 8.2	229	5.29 \pm 0.36	36.6 \pm 2.4	14.5 \pm 1.4	1.28 \pm 0.18
2.00	93.0 \pm 7.3	191	4.87 \pm 0.38	28.7 \pm 2.1	17.0 \pm 1.8	1.51 \pm 0.23
3.00	61.4 \pm 5.9	191	3.22 \pm 0.31	14.5 \pm 1.5	22.2 \pm 3.2	1.97 \pm 0.35
4.00	82.3 \pm 6.4	382	2.16 \pm 0.17	7.72 \pm 0.80	27.9 \pm 3.6	2.48 \pm 0.41
5.00	45.4 \pm 4.7	382	1.19 \pm 0.12	2.76 \pm 0.15	43.0 \pm 5.0	3.83 \pm 0.60

Table A3. Argon ion (330 MeV/amu) Enhancement of TK-Transformation of POT-TK5 Transfected (1.71 $\mu\text{g}/100\text{mm}$ dish) RAT-2 Cells

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	99.4 \pm 7.6	191	5.20 \pm 0.40	52.6 \pm 3.0	9.9 \pm 0.9	1.00 \pm 0.13
0.50	90.7 \pm 7.3	191	4.75 \pm 0.38	35.8 \pm 2.6	13.3 \pm 1.4	1.34 \pm 0.19
1.00	77.9 \pm 6.7	191	4.08 \pm 0.35	25.3 \pm 2.0	16.1 \pm 1.9	1.63 \pm 0.24
1.50	51.1 \pm 5.4	191	2.67 \pm 0.28	15.3 \pm 1.6	17.6 \pm 2.6	1.77 \pm 0.31
2.00	70.6 \pm 5.9	382	1.85 \pm 0.15	6.95 \pm 0.75	26.6 \pm 3.6	2.69 \pm 0.45
2.50	40.5 \pm 4.5	382	1.06 \pm 0.12	2.85 \pm 0.15	37.2 \pm 4.6	3.77 \pm 0.59

Table A4. X-rays Enhancement of TK-Transformation of POT-TK5 Transfected (10.8 μ g/100 mm dish) RAT-2 Cells

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	118.8 \pm 4.5	87	13.7 \pm 0.5	42.9 \pm 1.7	31.9 \pm 1.8	1.00 \pm 0.08
1.00	115.4 \pm 6.8	105	11.0 \pm 0.7	31.9 \pm 1.9	34.6 \pm 2.9	1.08 \pm 0.11
2.00	167.0 \pm 8.2	157	10.6 \pm 0.5	19.2 \pm 1.2	55.3 \pm 4.4	1.73 \pm 0.17
3.00	205.7 \pm 9.1	216	9.5 \pm 0.4	10.6 \pm 0.5	89.5 \pm 6.0	2.80 \pm 0.25
4.00	169.7 \pm 8.2	230	7.4 \pm 0.4	6.70 \pm 0.37	110.0 \pm 8.1	3.45 \pm 0.32
6.00	171.0 \pm 8.7	363	4.7 \pm 0.2	2.57 \pm 0.11	183.6 \pm 12.2	5.76 \pm 0.50

Table A5. X-ray Enhancement of A-Gene Transformation of POT-TK5 Transfected (10.8 $\mu\text{g}/100$ mm Dish) RAT-2 Cells.

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	17.2 \pm 0.9	87	1.97 \pm 0.10	42.9 \pm 1.7	4.61 \pm 0.31	1.00 \pm 0.10
1.00	19.4 \pm 1.3	105	1.84 \pm 0.12	31.9 \pm 1.9	5.80 \pm 0.51	1.26 \pm 0.14
2.00	26.5 \pm 1.7	157	1.69 \pm 0.11	19.2 \pm 1.2	8.78 \pm 0.78	1.90 \pm 0.21
3.00	61.4 \pm 3.6	432	1.42 \pm 0.08	10.6 \pm 0.5	13.36 \pm 1.04	2.90 \pm 0.31
4.00	67.2 \pm 3.8	576	1.17 \pm 0.07	6.70 \pm 0.37	17.41 \pm 1.37	3.78 \pm 0.39
6.00	89.0 \pm 4.3	1087	0.82 \pm 0.04	2.57 \pm 0.11	31.83 \pm 2.06	6.90 \pm 0.65

Table A6. X-Ray Enhancement of TK-Transformation of PTK2 Transfected (20 μ g/100mm dish) RAT-2 Cells.

Dose (Grays)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	211.2 \pm 13.1	215	9.82 \pm 0.60	62.7 \pm 2.4	15.7 \pm 1.1	1.00 \pm 0.10
1.00	271.6 \pm 14.7	240	11.32 \pm 0.61	52.7 \pm 3.0	21.5 \pm 1.7	1.37 \pm 0.15
2.00	228.0 \pm 13.5	274	8.32 \pm 0.49	39.2 \pm 3.4	21.2 \pm 2.2	1.35 \pm 0.17
3.00	316.0 \pm 15.9	416	7.60 \pm 0.38	21.0 \pm 2.0	36.2 \pm 3.9	2.31 \pm 0.30
4.00	178.6 \pm 12.0	266	6.71 \pm 0.45	13.5 \pm 0.9	49.7 \pm 4.8	3.17 \pm 0.38
5.00	147.4 \pm 10.9	264	5.58 \pm 0.41	7.20 \pm 0.5	77.5 \pm 7.6	4.94 \pm 0.60

Table A7. Ultraviolet Enhancement of TK-Transformation of PTK2 Transfected (13.7 $\mu\text{g}/100$ mm dish) RAT-2 Cells.

Dose (Joules/m ²)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	54.2 \pm 6.6	362	1.50 \pm 0.18	71.5 \pm 4.0	2.09 \pm 0.28	1.00 \pm 0.19
0.72	58.2 \pm 6.8	360	1.62 \pm 0.19	64.4 \pm 3.8	2.51 \pm 0.33	1.20 \pm 0.23
1.44	54.0 \pm 6.6	366	1.48 \pm 0.18	52.2 \pm 3.0	2.83 \pm 0.38	1.35 \pm 0.26
2.16	44.6 \pm 6.0	386	1.16 \pm 0.16	40.3 \pm 2.0	2.87 \pm 0.41	1.37 \pm 0.27
2.88	44.8 \pm 6.0	411	1.09 \pm 0.15	31.7 \pm 1.8	3.44 \pm 0.50	1.65 \pm 0.33
3.60	79.4 \pm 8.0	712	1.12 \pm 0.11	19.0 \pm 0.9	5.86 \pm 0.66	2.80 \pm 0.49
4.32	86.4 \pm 8.3	981	0.88 \pm 0.08	12.9 \pm 1.4	6.83 \pm 0.95	3.27 \pm 0.63

Table A8. Ultraviolet Enhancement of SV40 Viral DNA Transfected (11.1 $\mu\text{g}/100$ mm dish)
RAT-2 Cells.

Dose (Joules/m ²)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0.00	314.7 \pm 20.5	291	10.8 \pm 0.7	70.5 \pm 4.4	15.3 \pm 1.4	1.00 \pm 0.13
0.72	336.3 \pm 21.2	268	12.5 \pm 0.8	74.5 \pm 4.7	16.8 \pm 1.4	1.10 \pm 0.14
1.44	342.0 \pm 21.4	271	12.6 \pm 0.8	56.7 \pm 3.5	22.3 \pm 1.9	1.45 \pm 0.18
2.16	320.3 \pm 20.7	294	10.9 \pm 0.7	43.2 \pm 2.7	25.2 \pm 2.2	1.65 \pm 0.21
3.60	199.4 \pm 12.6	282	7.1 \pm 0.4	24.2 \pm 1.5	29.2 \pm 2.4	1.91 \pm 0.23
4.32	143.7 \pm 9.1	259	5.6 \pm 0.4	17.9 \pm 1.1	30.1 \pm 3.1	1.97 \pm 0.27

Table A9. PTK2 Titration using LTK⁻ Carrier DNA (12.0 µg/100 mm dish) Transfected into RAT-2 cells.

Concentration of PTK2 DNA (ng/dish)	Average	Number of	Transformants		Number of
	Number of Transformants Per Dish	Cells plated Per Dish ($\times 10^{-3}$)	Per Cell Plated ($\times 10^4$)	Percentage Survival	Transformants Per Survivor ($\times 10^4$)
0	0	560	0	71.4 ± 4.2	0
45	23.8 ± 4.0	575	0.41 ± 0.07	72.4 ± 3.9	0.57 ± 0.10
90	39.0 ± 5.1	535	0.73 ± 0.10	67.6 ± 3.7	1.08 ± 0.16
150	52.0 ± 5.9	376	1.38 ± 0.16	77.0 ± 4.2	1.80 ± 0.22
300	85.9 ± 7.0	356	2.41 ± 0.20	76.3 ± 4.1	3.16 ± 0.31
450	91.7 ± 7.2	304	3.02 ± 0.24	77.4 ± 4.2	3.90 ± 0.38
600	132.3 ± 9.4	332	3.98 ± 0.28	76.7 ± 4.1	5.20 ± 0.47
750	120.0 ± 8.3	284	4.29 ± 0.29	76.8 ± 4.1	5.50 ± 0.51
900	148.2 ± 7.7	374	3.96 ± 0.21	73.5 ± 4.0	5.40 ± 0.40

Table A10. X-Ray Enhancement (5.00 Grays) of PTK2 titration using LTK- Carrier DNA (12.0 $\mu\text{g}/100$ mm dish) Transfected into RAT-2 Cells.

Concentration of PTK2 DNA (ng/dish)	Average Number of Transformants Per Dish	Number of Cells plated Per Dish ($\times 10^{-3}$)	Transformants Per Cell Plated ($\times 10^4$)	Percentage Survival	Number of Transformants Per Survivor ($\times 10^4$)	Enhancement Ratio
0	0	300	0	2.3 ± 0.2	0	
45	11.8 ± 1.8	546	0.22 ± 0.03	2.1 ± 0.3	10.3 ± 2.0	18.0 ± 4.7
90	20.4 ± 2.3	468	0.44 ± 0.05	2.4 ± 0.3	18.9 ± 2.9	16.8 ± 3.6
150	14.6 ± 2.0	341	0.43 ± 0.06	2.0 ± 0.2	21.5 ± 3.7	12.0 ± 2.6
300	23.1 ± 2.5	339	0.68 ± 0.07	1.9 ± 0.1	35.4 ± 5.5	9.0 ± 2.1
450	12.4 ± 1.8	252	0.49 ± 0.07	1.4 ± 0.1	35.2 ± 7.4	11.2 ± 2.1
600	22.0 ± 2.4	313	0.70 ± 0.08	1.6 ± 0.1	44.8 ± 7.5	8.6 ± 1.7
750	21.3 ± 2.4	268	0.80 ± 0.09	2.6 ± 0.2	30.3 ± 4.7	5.5 ± 1.0
900	27.1 ± 2.7	313	0.87 ± 0.09	2.3 ± 0.1	37.9 ± 5.5	7.0 ± 1.1

APPENDIX B: REAGENTS AND THEIR PREPARATION

I. STOCK SOLUTIONS

1.0 M Tris - Dissolve 121.1 g Tris base in 800 ml of water. Adjust the pH to the desired value by adding concentrated HCl. Allow the solution to cool to room temperature before adjusting final pH. Adjust volume to 1.0 liter. Sterilize by autoclaving.

0.5 M EDTA (pH 8.0) - Add 186.1 g of disodium ethylene diamine tetraacetate dihydrate and approximately 20 g NaOH pellets to 800 ml water. Stir vigorously with a magnetic stirrer. Adjust pH to 8.0 (the disodium salt of EDTA will not go into solution unless the pH is greater than 8.0), adjust volume to 1.0 liter. Sterilize by autoclaving.

5 M NaCl - Dissolve 292.2 g of NaCl in 800 ml H₂O. Adjust volume to 1.0 liter. Sterilize by autoclaving.

1 M MgCl₂ - Dissolve 203.3 g of MgCl₂·6H₂O in 800 ml of H₂O. Sterilize by autoclaving.

1 M DTT - Dissolve 3.09 g Dithiothreitol in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterilize by filtration. Store at -20°C. Never autoclave solutions with DTT in them.

3 M sodium acetate - Dissolve 408.1 g of sodium acetate trihydrate in 800 ml of water. Adjust pH to 5.2 with glacial acetic acid (not concentrated HCl!). Adjust volume to 1.0 liter. Sterilize by autoclaving.

Beta-Mercaptoethanol (BME) - Usually obtained as 14.4 M solution. Store in a dark bottle at 4°C.

10 per cent SDS - Dissolve 100 g of electrophoresis-grade sodium dodecyl sulfate (sodium lauryl sulfate) in 900 ml water. Heat to 68°C to assist dissolution. Adjust volume to 1.0 liter.

1 M Magnesium acetate - Dissolve 214.56 g of magnesium acetate tetrahydrate in 800 ml of water. Adjust volume to 1.0 liter.

Sterilize by filtration.

20 x SSC - Dissolve 175.3 g of NaCl and 88.2 g sodium citrate in 800 ml water. Adjust pH to 7.0 with a few drops of 10 N NaOH. Adjust volume to 1.0 liter. Sterilize by autoclaving.

Ethidium bromide (10 mg/ml) - Add 1 g of ethidium bromide to 100 ml of water (Wear gloves, ethidium bromide is a powerful mutagen!). Stir on magnetic stirrer for several hours for complete dissolution. Store in container wrapped in aluminum foil and store at 4°C.

Denhardt's Solution (50x) - Add 5g Ficoll, 5 g polyvinylpyrrolidone, and 5 g BSA (Pentax Fraction V) to 500 ml water. Filter through a disposable Nalgene filter. Store at 4°C. Dissolve at low heat on stirrer, spin for 2K rpm for 15 min.

TE - 1 mM EDTA (pH 8.0)

10 mM Tris.Cl (at desired pH)

For most DNA solutions, TE pH 8.0 is recommended.

Gel Soak I - 4 liters solution : 32 g NaOH, 140.3 g NaCl

Gel Soak II - 4 liters solution 484.4 g Tris Base, 140.3 g NaCl PH to 7.4 with approximately 210 ml concentrated HCl, adjust volume to 4 liters.

II. BUFFERS

Electrophoresis Buffer (20 x E) - 4 liters

<u>Working Concentration</u>	<u>Amount for 4 liters</u>
0.8 M Tris	387.5 g
0.1 M Sodium Acetate	54.4 g
0.02 M EDTA	29.8 g

pH to 7.8 with approximately 125 ml glacial acetic acid.

Loading Buffer - 6x

0.25 per cent bromophenol blue

0.25 per cent xylene cyanol

30 per cent glycerol in water. Store at 40C.

Hirt Lysis Buffer

30 ml 10 per cent SDS

5 ml 1 M Tris (pH 7.9)

10 ml 0.5 M EDTA

Water up to 500 ml total volume

III. PHENOL PREPARATION AND PHENOL EXTRACTION

It is often necessary to remove proteins from nucleic acid solutions after DNA manipulations. For most simple DNA purification steps extraction with a phenol mixture is sufficient. When removing high molecular weight DNA from cell lysates it is usual to first remove most of the protein by digesting with proteinase K, a proteolytic enzyme, then extracting with organic solvents. In this dissertation, phenol refers to a 50:48:2 mixture of phenol: chloroform: isoamyl

alcohol. The phenol and the chloroform denatures the protein, while the isoamyl alcohol reduces foaming during the extraction and facilitates the separation of the organic and aqueous phases.

A. PHENOL PREPARATION - Liquified phenol is made 0.1 percent (w/v) in 8-Hydroxyquinoline, and then extracted twice with an equal volume of 1.0 M Tris (pH 8.0). I found that the best way to separate the phases, was to spin 250 ml of the mixture in 400 ml-centrifuge bottles in a GS3 rotor for five minutes at 5000 rpm. The aqueous layer is aspirated off. After the final extraction phenol, chloroform, and isoamyl alcohol are mixed at 50:48:2 parts. This final phenol mixture is equilibrated with an equal volume of 0.1 M Tris (pH 8.0), and 0.2 percent beta-mercaptoethanol. The equilibrated phenol solution is stored at 4°C in a dark brown jar.

B. EXTRACTION WITH PHENOL

1. Mix aqueous DNA sample with an equal volume of phenol in a polypropylene tube with a plastic cap. Phenol will dissolve polycarbonate tubes. If using an Eppendorf tube, 0.50 ml is the maximum volume of the DNA sample.
2. Centrifuge for 3 minutes at 1600 g or for 2 minutes in an Eppendorf centrifuge at room temperature.
3. Use a pipette to transfer the upper aqueous phase to a fresh polypropylene tube. For small volumes use an automatic pipettor fitted with a disposable tip. Discard the organic phase and the interface.

4. Repeat steps 1, 2, and 3.
5. To rid the aqueous phase of the residual phenol, add an equal volume of ether. Do this in a flow hood, since anhydrous ether is extremely volatile and explosive.
6. Spin in Eppendorf for 10 seconds at room temperature.
7. Using pipette pull off the upper, ether layer and discard in a waste vessel.
8. Repeat steps 5, 6, and 7.
9. To rid the aqueous solution of the residual ether, place open tubes in an incubator for 5 minutes.
10. Recover the DNA by precipitation with ethanol as described in Appendix B. IV.

IV. CELL CULTURE MEDIA AND SOLUTIONS

A. Dulbecco's Modified Eagles Medium (DME)

1. For 10,000 ml of medium add in a Millipore pressure vessel:

NaHCO ₃	37 grams
Pen-Strep (10,000 U/ml)	100 ml
DME powder (Gibco)	137 grams
Water, distilled	9,500 ml
2. Bubble CO₂ into outlet valve, till powder dissolved, and the media is amber color—30 minutes.
3. Filter through Millistak No. MSGS-05CHZ (Millipore) filter into autoclaved 500 ml bottles.

4. The Millistak filter is also capable of filtering 10 liters of media containing 10 per cent sera.

B. HAT (100 X) Concentrate

- For 100 mls concentrate, add to 100 mls water:
 - 0.15 g Hypoxanthine
 - 0.01 g Aminopterin
 - 0.05 g Thymidine
- Add 0.8 ml of 10 N NaOH (0.08 M). The NaOH clears the solution.
- Filter sterilize, store at 4°C, in bottle covered with aluminum foil.

C. Phosphate Buffered Saline (PBS)

- For 1000 ml PBS, add

8 gm NaCl	0.1 g CaCl ₂ dihydrate
0.2 g KCl	0.1g MgCl ₂ 6H ₂ O
0.2 g KH ₂ PO ₄	
- Dissolve 0.1 g Na₂HPO₄ separately in 100 ml water.
- Add all dissolved reagents, adjust volume to 1000 ml.
Filter sterilize.

D. Tris Buffered Saline (TBS)

- For 1000 ml TBS, add

3 g Tris base'	0.1 g CaCl ₂ dihydrate
8 g NaCl	0.1 g MgCl ₂ 6H ₂ O
	0.38 g KCl

2. Dissolve 0.1 g Na_2HPO_4 separately in 100 ml water.
3. Add all dissolved reagents adjust volume to 800 ml with water.
4. Solution is cloudy, but clears when adding HCl to pH 7.5.
5. Adjust volume to 1000 ml. Filter Sterilize.

E. Trypsin - 0.05 per cent solution.

1. For a final volume of 1000 ml, add: 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , 1.15 g Na_2HPO_4 (dissolved separately in 100 ml), 2 ml EDTA (0.5 M)
2. Adjust volume to 800 ml and pH to 7.2 with NaOH. Adjust volume to 1000 ml.
3. Pour 50 ml into 100 ml screw-capped bottles, autoclave at liquid cycle.
4. After cooled, add 1 ml of concentrated sterile 2.5 per cent Trypsin (Gibco). Store at -20°C .

V. ETHANOL PRECIPITATION

This is the easiest and most widely used method for concentrating DNA from aqueous solutions. The precipitate of DNA, which is formed at low temperatures in the presence of moderate concentrations of monovalent cations, is recovered by centrifugation and redissolved in an appropriate buffer.

1. Estimate the volume of the DNA solution.
2. Make 0.1 M in NaCl, by adding 1/50 volume 5 M NaCl, shake well.
3. Add exactly 2 volumes of ethanol (95 percent) and mix well.

4. Chill at -20°C for 30–60 minutes; -70°C for 15–30 minutes; or at -78°C in a dry ice/ethanol bath for 5 minutes.
5. Centrifuge at 0°C . For most purposes, 10 minutes in an Eppendorf centrifuge or at 12,000 g is sufficient.
6. Discard the supernatant. Traces of supernatant may be removed by brief treatment (1–2 minutes) in a vacuum desiccator or lyophilizer.
7. Dissolve the DNA pellet (which is often invisible) in the desired volume of buffer. For storage of DNA samples, TE at pH 8.0 is used. The sample can be heated to 37°C for five to 15 minutes to assist in dissolution.

VI. PREPARATION AND USE OF DIALYSIS TUBING

A. PREPARATION

1. Cut tubing into pieces of convenient length.
2. Boil for 10 minutes in a large volume of 2 percent sodium bicarbonate and 1mM EDTA.
3. Rinse the tubing thoroughly in distilled water.
4. Repeat steps 2 and 3.
5. Boil tubing for 10 minutes in distilled water.
6. Rinse the tubing thoroughly in distilled water.
7. Store tubing in 70 percent ethanol at 4°C .

B. USE OF DIALYSIS TUBING

1. Wash tubing inside and out with distilled water. Always use gloves to handle tubing—RNAse and DNAse contamination is present in your hands.

2. Knot one end of tubing, add sample to be dialyzed, and knot the other end.
3. Dialyze in large volume of appropriate buffer.

VII. CHROMATOGRAPHY THROUGH SEPHADEX G-50.

Sephadex G-50 is used to separate high-molecular weight DNA from smaller molecules such as unincorporated, labeled deoxynucleotide triphosphates, or salts) by gel filtration.

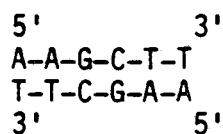
- A. PREPARATION OF SEPHADEX G-50. Slowly add 30 g of Sephadex G-50 (medium) to 250 ml of TE (pH 8.0) in a 500-ml beaker or bottle. Make sure the powder is well dispersed and wetted. Let stand overnight at room temperature. Decant the supernatant and replace with an equal volume of TE (pH 8.0). Store at 4°C in a screw-capped bottle.
- B. SPIN COLUMN PROCEDURE
 1. Add 9 ml pack volume of Sephadex G-50 in a 10 ml quik-sep disposable column with sintered disc. The column is designed so that it will not run dry if eluent not administered.
 2. Spin column in a 14 ml polypropylene test tube for 5 minutes at 1000 K rmp and 5 minutes at 3000 rpm to pack column and spin out buffer.
 3. Decant out the spun-out buffer and place an Eppendorf tube with its cap removed in the bottom of the test tube. Place the spun column back in the test tube.

4. Add 0.100 ml of DNA sample to the dry column.
5. Spin for 5 minutes at 3000 rpm. The unincorporated triphosphates remain in the column, and should be carefully discarded. The DNA is collected from the decapped Eppendorf tube (a volume approximately 0.70 ml).

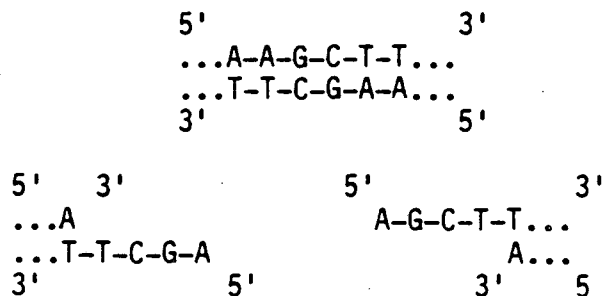
APPENDIX C: DNA MANIPULATIONS

I. RESTRICTION ENDONUCLEASES. Restriction endonucleases are enzymes isolated chiefly from prokaryotes that recognize specific sequences within double-stranded DNA. These enzymes fall into three groups. Type-I and type-III enzymes carry two functions in their proteins—methylation and cleavage. Both classes recognize unmethylated sequences in DNA, but type-I enzymes cleave randomly while type-III enzymes cut DNA at specific sites.

Type-II restriction endonucleases contain no associated methylase activity and cut DNA within or near to their particular recognition sequences. These sequences are four to six nucleotides in length with a two-fold axis of symmetry—a palindrome. The enzyme Hind III, for example, recognizes the hexanucleotide sequence:



Like many other restriction endonucleases, Hind III does not exactly cleave at the axis of dyad symmetry but at positions four nucleotides apart in the two DNA strands:



The staggered cleavage produces DNA fragments with protruding cohesive 5' tails. Any of the Hind III termini can base pair with each other. DNA molecules cleaved at sites with the same recognition sequence can be joined by DNA ligase to form new recombinant molecules.

Many restriction endonucleases like Hind III, Eco RI, Bam HI, etc., generate DNA fragments with protruding 5' tails; others (e.g., Pst I, Kpn I) generate fragments with 3' protruding cohesive termini; whereas, still others (e.g., Bal I, Pvu II, Sma I) cleave at the axis of symmetry to produce blunt-ended fragments.

Unique stretches of DNA contain a myriad of recognition sequences for a variety of enzymes. By cutting DNA with different enzymes a physical map of the DNA can be generated that acts as a signature of the DNA sequence.

A physical map of a DNA sequence is produced by monitoring the cleavage pattern of fragments separated in an electrophoresis gel. In an electric field DNA fragments suspended in an electrolyte solution (pH greater than 7.6) will migrate toward the positive cathode. In electrophoresis buffer all DNA fragments have the same approximate mass to charge ratio. If a cross-linked matrix (e.g., polyacrylamide or agarose) is placed in the migration path, the DNA fragments will separate according to size—producing distinct bands.

RESTRICTION ENDONUCLEASES

Figure A.1.

Bam HI

Bacillus amyloliquefaciens H

↓
GGATCC
CCTAGG
▲

Eco RI

Escherichia coli RY13

↓
GAATTC
CTTAAG
▲

Hpa II

Haemophilus parainfluenzae

↓
CCGG
GGCC
▲

Kpn I

Klebsiella pneumoniae OK8

↓
GGTACC
CCATGG
▲

Pvu II

Proteus vulgaris

↓
CAGCTG
GTCGAC
▲

In practice, DNA is cut with a variety of enzymes and then loaded into a slot made in an agarose gel. The gel is submerged in electrophoresis buffer and then an electric field is produced by immersing platinum electrodes. The DNA migrates into the gel and the fragments are fractionated according to size. After electrophoresis the gel is submerged in a bath of ethidium bromide (1 ug/ml). Ultraviolet light absorbed by ethidium bromide (300 nm) or absorbed by DNA (260 nm) and the energy transferred to the intercalating ethidium bromide is re-emitted as fluorescence at 590 nm.

A. PROCEDURE FOR CUTTING DNA

The optimum restriction buffer formula for each enzyme is supplied by the vendor. But an all-purpose reaction buffer exists, Tris-acetate (TA) buffer. Every enzyme should be tested in this buffer before experimentation. A 10 fold concentrate (10xTA) consists:

- 0.33 M Tris-acetate (pH 7.9)
- 0.66 M potassium acetate
- 0.10 M magnesium acetate
- 0.005 M dithiothreitol
- 1 mg/ml bovine serum albumin (BSA Pentax Fraction V) (I have omitted the BSA with good results).

The 10 x stock should be stored frozen at -20°C in aliquots.

Setting up the Digest - for a typical 50 μl reaction.

1. Mix water with the DNA solution in an Eppendorf tube to give a final volume of 45 μl . The DNA concentration should be between 0.2-1 μg for 50 μl final volume;

2. Add 5 μ l of 10x TA buffer (or appropriate digestion buffer).
3. Add 1 unit of restriction enzyme, digest for 1 hour at 37°C.
4. Stop reaction with 1 μ l of 0.5 M EDTA.
5. If the restricted DNA is to be purified, phenol extract, and then ethanol precipitate (Appendix B).
6. If the DNA is to be analyzed directly on a gel, add 10 μ l of gel-loading dye, mix by vortexing, and load an aliquot of the digest into the gel slot.
7. It is convenient throughout manipulations to briefly (3 seconds) spin the Eppendorf tubes to bring down the solutions that are clinging to the sides of the tube.
8. For more details see Maniatis, et al., 1982.

B. RUNNING GEL - Follow the instructions of casting a agarose electrophoresis gel given by the manufacturer. Seakem ME Agarose contains less contaminants than the average grade of agarose.

1. Submerge the gel in 1X E buffer (made from 20x E buffer, Appendix B), leaving at least 1 cm over gel.
2. Load digest-dye solution, so that slot is only one-half full.
3. Connect electrodes, and electrophorese.
4. Submerge gel in a 1:10,000 dilution of 10 mg/ml ethidium bromide (Wear gloves with all manipulations with ethidium bromide). Stain for 15 minutes.
5. Carefully place gel on UV-box. Observe gel with UV-protective glasses.

II. LIGATION - To simplify manipulations, ligation may be performed directly in the reaction mixture with heat inactivated restriction endonuclease. By adding ATP to the mixture, the DNA ligase may be added to the reaction directly.

A. Reaction

1. Stop restriction endonuclease digest by heating reaction for 5 minutes at 68°C.
2. Mix DNA to be ligated in a total volume of 17 μ l in TA buffer. This mixture may contain 0.2-2 μ g DNA.
3. Add 2 μ l of a 0.1 M ATP solution (store frozen).
4. Add 1 μ l of concentrated ligase.
5. Incubate at room temperature for 1 hour.
 - a. For recircularization for blunt ends, leave overnight at room temperature.
 - b. For recircularization for sticky ends, leave overnight at 4°C.
6. Reaction terminated by incubating at 68°C for five minutes.

B. Reaction may be monitored by electrophoresis.

C. 10 μ l of reaction mixture is sufficient to transform 0.100 ml of competent bacteria.

III. NICK TRANSLATION--LABELING DNA

E.coli DNA polymerase I (pol I) adds nucleotide residues to the 3'hydroxy end of DNA that is created when one strand of double-stranded DNA is nicked. The enzyme is also able to excise

nucleotides from the 5' side of the nick by virtue of its 5' to 3' exonuclease activity. The process of replacing nucleotides after their elimination moves the nick in a 5' to 3' direction and is called nick translation. If radioactive nucleotides are present during nick-translation, it is possible to prepare ^{32}P -labeled DNA with a specific activity greater than 10^8 cpm/ μg (Maniatis, et al., 1975; Rigby et al., 1977).

A. MATERIALS.

1. Radioactive triphosphates (Amersham): 410-635 Ci/mmole
 - a. Deoxyadenosine 5'(a- ^{32}P)triphosphate (PB. 10164)
 - b. Deoxycytidine 5'(a- ^{32}P)triphosphate (PB. 10165)
 - c. Deoxyguanosine 5'(a- ^{32}P)triphosphate (PB. 10166)
 - d. Thymidine 5'(a- ^{32}P)triphosphate (PB. 10167)
2. 10 x Nick translation Buffer (10x NTB) - store at -20°C .
 - a. 0.5 M Tris-Cl (pH 7.2)
 - b. 0.1 M MgSO_4
 - c. 1 mM dithiothreitol
 - d. 0.5 mg/ml bovine serum albumin (BSA Pentax Fraction V)
3. DNA polymerase I (Bethesda Research Lab.; New England Biolab.)
4. DNase I: Worthington 1 mg/ml in 0.15 M NaCl and 50 percent glycerol, store at -20°C .

B. REACTION - reaction is performed in a 1.5 ml Eppendorf tube.
Use adequate radiation shielding.

1. Add 1 μg of DNA into Eppendorf tube

2. Add 10 μ l of 10X nick translation buffer.
3. Add 6 μ l each of the four radioactive dNTPs
4. Add Water to made volume at 98 μ l
5. Dilute DNase stock 1:10,000 in nick-translation buffer.
Add 1 μ l immediately to reaction mixture.
6. Add 1 μ l of DNA polymerase 1.
7. Incubate at 16°C for 30 minutes in a styrofoam container.
8. Stop reaction by adding 2 μ l of 0.5 M EDTA.
9. Separate the nick-translated DNA from unincorporated dNTPs
by centrifugation through a small column of Sephadex G-50
(Appendix B,VI).

IV. DNA FRAGMENT ISOLATION - It is often convenient or necessary to isolate specific DNA restriction fragments for sub-cloning or labeling. The procedure presented is the most successful I have found for speed, yield, and the highest retained biological activity. DNA will bind to glass beads at high ionic strength. DNA fragments fractionated on agarose gels, are isolated by slicing gel with fregment, dissolving the agarose in sodium iodide and binding the DNA fragment to glass beads (Vogelstein and Gillespie, 1979).

A. Preparation of Glass beads.

1. Silica-325 Mesh a powdered flint glass is obtainable from ceramic stores.
2. Suspend a 250 ml volume of silica-325 in water to fill a 500 ml beaker.
3. Stir with magnetic stirrer for 1 hr at room temperature;

4. Let silica settle for one hour and remove upper fines by siphon, centrifuge them in Sorvall centrifuge
5. Resuspend pellet in 100–200 ml water.
6. Add equal volume nitric acid
7. Bring close to boil, while stirring (in Flow hood).
8. Wash four times with water, pellet after every wash.
9. Store as a 50 percent slurry in water at 4°C.
10. 250 ml volume of powder yields about 20 g fines, which is enough to isolate approximately 40 mg of DNA fragments.

B. Sodium Iodide Solution

1. Add 90.8 g NaI, 1.5 g Na₂SO₃, to 100 ml water, dissolve.
2. Filter through a disposable Nalgene filter, then add 0.5 g Na₂SO₃. Store at -20°C, in bottle covered with Al foil.

- C. Ethanol Wash Solution: 10 mM Tris (pH 7.5),
0.1 M NaCl, in 50 per cent ethanol. Store at -20°C.

D. BEAD ISOLATION PROTOCOL - use only plastic tubes.

1. Fractionate DNA digest on agarose gel, stain with EtBr, carefully cut out gel slice with DNA fragment.
2. Weigh slice, then grind through a syringe without a needle.
3. Dissolve agarose in NaI solution: 2ml/g gel, in an Eppendorf tube. Incubate with occasional mixing at 37°C until totally dissolved.

4. Shake glass bead slurry until all powder is in suspension. Add appropriate aliquot to dissolved gel and let DNA adsorb to the beads for 60 minutes on ice. 20 μ l of slurry is plenty for 15 μ g DNA in 1.5 ml. Occasionally invert tube to keep glass in suspension. (Adsorption can proceed overnight on ice.)
5. Spin out pellet - 15 seconds in microfuge.
6. Wash pellet with 10 volumes NaI Solution, at 0-4°C.
7. Repeat steps 5 and 6, two times.
8. Wash pellet with 10 volumes of cold ethanol wash solution.
9. Pellet.
10. Repeat steps 8 and 9, two times.
11. Dessicate pellet briefly in vacuum dessicator.
12. Resuspend pellet in two volumes of TE (pH 8.0) and incubate for 30 minutes at 60-68°C.
13. Pellet in microfuge.
14. Pipette out the TE solution with the eluted DNA.
15. Repeat steps 12, 13, 14.
16. Pool the two extractions, and spin one more time to pellet out any residual beads.
17. The DNA is now in TE, and is ready for nick-translation or restriction analysis. Store at 4°C.

V. FILLING IN PROTRUDING ENDS TO BLUNT ENDS.

It is often not possible to join two desired fragments of DNA

which have appropriate termini for ligation. For instance a DNA fragment with a 5' protruding end (e.g., BAM HI) can not be ligated to a fragment with a 3' protruding end (e.g., KPN I) nor to a blunt ended fragment (e.g., PVU II). Protruding 5' ends are filled using the DNA polymerizing activity of the Klenow fragment of E. coli polymerase I. Protruding 3' ends are made blunt-ended by the 3' exonuclease activity of T4 DNA polymerase.

A. Preparing stock solutions of Dextroribonucleotide Triphosphates.

1. Dissolve the appropriate dNTP in double-distilled water directly in the shipping vial, to an expected concentration of 10 mM.
2. pH to 7.0 by use of 0.05 M Tris (pH 7.0), and pH paper.
3. Dilute an aliquot of dNTP approximately 1:100, and determine concentration by optical density using these extinction coefficients:

BASE	WAVELENGTH	EXTINCTION COEFFICIENTS $e(\text{mM}^{-1}\text{cm}^{-1})$
A	259	15.4
C	271	9.1
G	253	13.7
T	260	7.4

B. Filling in 5' ends

1. Set up the following Reaction:
 - a. Restriction fragment (up to 2 μ g DNA in 10 μ l)
 - b. 2 mM solution of all four dNTPs 4 μ l
 - c. 10 x NTB (Nick Translation Buffer) 10 μ l
 - d. Water to total volume 100 μ l
2. Add 5 units of Klenow fragment. Mix and incubate for 15-30 minutes at 17°C. Heat to 68°C for five minutes to inactivate the enzyme.
3. Unincorporated dNTPs may be extracted from the blunt-ended DNA by spinning through a Sephadex G-50 column.

C. Blunt-ending 3' ends.

1. Set up this reaction:
 - a. Restriction fragment (up to 4 μ g in 20 μ l)
 - b. 10 X TA buffer
 - c. 2mM solution of all four dNTPs 5 μ l
 - d. water to total volume 100 μ l
 - e. T4 DNA polymerase (approx. 8 u) 3 μ l
2. Incubate for 5-10 minutes at 37°C.
3. Add 1 μ l of 0.5 M EDTA.
4. Phenol extract once, ether extract once.
5. Purify blunt ended fragment from unincorporated dNTPs by spinning through Sephadex G-50 column.

D. END-LABELING DNA FRAGMENTS WITH PROTRUDING TERMINI.

The same protocols may be employed to end-label protruding termini of DNA fragments. Substitute approximately 2 μCi of the appropriate labeled dNTP for the 'cold' one. Perform reaction, then chase with cold dNTP to allow reaction to reach completion.

APPENDIX D: DNA EXTRACTION TECHNIQUES

This appendix discusses the techniques used to extract DNA from mammalian cells and bacteria. These techniques include large scale isolation of bacterial plasmid DNA for genetic engineering and preparation of high molecular weight DNA from mammalian cells for Southern analysis. While many of these techniques have been documented, most have been modified by myself and my colleagues.

I. EXTRACTION OF BACTERIAL DNA.

A. "Cracking" Reaction to screen for Gross changes in plasmid DNA. In some instances the genetic engineering procedures include screening bacterial plasmids for gross deletions or insertions, or sizing of an unknown plasmid. This technique is much faster than the "Mini-prep" in section B.

1. Grow bacterial clones overnight in LB broth with appropriate antibiotic.
2. Pellet 1.00 ml of overnight in a 1.5 ml Eppendorf tube, spin for 30 seconds.
3. Aspirate supernatant.
4. Add 200 μ l of phenol solution and 200 μ l of 1X loading buffer. Vortex to complete dissolution.
5. Spin in microfuge for 2 minutes. Three distinct phases may be seen: the lower organic layer, the upper aqueous layer, and the protein interface.

6. Carefully load an aliquot of the upper-aqueous layer directly into an agarose gel and electrophorese. After staining, the plasmid can be easily seen.

B. BACTERIAL MINI-PREP

This technique is suitable for recovery of approximately 1 μ g of plasmid DNA from 1.5 ml of an overnight bacterial suspension. One microgram of DNA is sufficient for about five restriction endonuclease reactions. This mini-prep technique is used to determine the physical map of a series (in one day at least 100)preps.

1. Materials

- a. At least 1.5 ml of overnight suspensions of bacteria.
- b. Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0.
- c. Solution II: 0.2 N NaOH, 1 per cent SDS (make fresh).
- d. Solution III: 29.4 g potassium acetate, 5 ml of 90 per cent formic acid, water to 100 ml.
- e. Acetate-MOPS: 0.1 M sodium acetate, 0.05 M MOPS (morpholinopropanesulfonic acid, Sigma), pH 8.0 with NaOH.

2. Protocol

- a. Pour 1.5 ml of culture into Eppendorf tube, spin 1 minute in microfuge.
- b. Remove media by aspiration.

- c. Suspend pellet in 100 μ l of Solution I, incubate at room temperature for five minutes.
- d. Add 200 μ l of freshly prepared Solution II. Close the top of tube and mix contents by inverting tube two or three times. Store tube on ice for five minutes.
- e. Add 150 μ l of cold Solution III. Vortex gently, store on ice for five minutes.
- f. Centrifuge for 5-10 minutes in microfuge at 4°C.
- g. Carefully transfer supernatant to fresh tube.
- h. Phenol extract once.
- i. Ether extract once.
- j. Ethanol precipitate DNA.
- k. Resuspend dessicated pellet in 100 μ l of Acetate-MOPS and ethanol precipitate.
- l. Resuspend pellet in TE (pH 8.0), 50 μ l.
- m. 10 μ l is sufficient to be cut with desired restriction endonuclease.

C. LARGE SCALE PREP

To obtain large yields of plasmid DNA from bacteria I employed three basic steps: a) growth of the bacteria and amplification of the plasmid DNA, b) harvesting and lysis of the bacteria, c) and purification of the plasmid DNA. The procedure described has been modified from Birboim and Doly (1979).

Materials and Protocol

1. 10 ml of a fresh overnight of bacteria (HB101) is inoculated into 500 ml of LB media with antibiotic in a 2.0 liter Erhlemeyer flask.
2. The flask is shaken vigorously at 37°C for about 3.0-3.5 hours.
3. When the optical density at 600 nm reaches 0.8 -1.0 125 mg of solid chloramphenicol is added. Continue shaking and incubation for 15-19 hours.
4. Harvest cells by centrifugation for 5 minutes at 5000 RPM in 400 ml bottles (fill only to 250 ml, use a Mettler P1200 for rapid balancing of bottles) in a GS3 rotor.
5. Aspirate all media from bottle (containing 500 ml pellet).
6. Resuspend pellet in 10 ml of Solution I (See Mini-Prep for these formulae). Incubate at room temperature for 10 minutes. (I found that lysozyme is not necessary for my strain of HB101).
7. 20 ml of freshly made Solution II is added with gentle swirling, incubate on ice for exactly five minutes.
8. Add 15 ml of cold Solution III, incubate on ice for 15 minutes.
9. Centrifuge for 30 minutes in GS3 at 8000 rpm.
10. Pour supernatant through a plastic funnel with cheese-cloth into a GSA centrifuge bottle. This should be approximately 45 ml of solution.

11. Add 27 ml (0.6 volume) of isopropyl alcohol to solution.
Place bottle in -20°C for 30 minutes.
12. Centrifuge in GSA rotor for 30 minutes, 4°C , at 8000 rpm.
13. Resuspend pellet in 10 ml (total volume) Acetate-Mops and Ethanol precipitate (20 ml Ethanol) in a 40 ml centrifuge tube that fits a SS34 rotor.
14. Centrifuge in SS34 for 10 minutes, 4°C , 10000 rpm.
15. Ethanol precipitate once more in Acetate-Mops.
16. Resuspend pellet in 2ml of water.
17. Add 2ml of LiCl solution (5M LiCl, 0.05 M MOPS, pH to 8.0 with NaOH) and place on ice for 15 minutes.
18. Spin in SS34 for 10 minutes at 10,000 rpm (this pellet contains 95 per cent of the ribosomal RNA and protein).
19. The supernatant is saved (4ml) and 8ml of ethanol is added. The solution is kept at 20°C for 15 minutes, and spun at 10,000 rpm, 4°C , for 10 minutes.
20. The pellet is resuspended in 10 ml acetate-MOPS and ethanol precipitated with 20 ml of ethanol.
21. Repeat step 20.
22. Dessicate pellet for 5 minutes in vacuum dessicator.
23. Resuspend pellet in TE pH 8.0, to a total volume of 7.4 ml.
24. Add 7.8 g reagent grade Cesium chloride. Mix well. Plasmid DNA can be separated from high molecular E.coli DNA by equilibrium centrifugation in cesium chloride gradients. Covalently closed, circular DNA binds much less of the

intercalating dye, ethidium bromide, than linear DNA. The dye-bound plasmid DNA will band at a higher density (1.55 g/ml) than the dye-bound linear DNA (Radloff, et al., 1967).

25. Add 0.4 ml of 10 mg/ml ethidium bromide.
26. Load 5 ml of DNA solution into each of two "Quick Seal Vertical Centrifuge Tubes," 13 x 51, (Beckmann polyallomer, P3-05-293, and balance tubes with rebanding solution (37 ml TE pH 8.0, 2.0 ml 10mg/ml ethidium bromide, 39 g CsCl).
27. Seal tubes and load into Beckman VTi65 rotor.
28. Centrifuge 4 hours, 62K rpm, 15°C,
29. Snip off the tops of the centrifuge tubes, and with a Pasteur pipette draw off the lower band of DNA plasmid.
30. Pool bands and place in new centrifuge tube, fill the rest of the volume with rebanding solution.
31. Recentrifuge under conditions in step 28.
32. Pull off band as before, and place in test tube.
33. Extract the ethidium bromide from the DNA by adding equal volume of sec-butanol. If during extraction process the cesium chloride precipitates out, add a drop or two of water to redissolve the salt.
34. Repeat the butanol extractions until the aqueous solution is colorless and does not fluoresce under UV irradiation.
35. The cesium chloride is removed by extensively dialyzing the DNA solution in TE buffer pH 8.0. For a 2ml volume, dialyze in three changes of one liter of TE at 4°C for at least one hour each change.

36. The concentration of the DNA is determined by optical density. An O.D. of 1.00 at 260 nm represents 50 $\mu\text{g/ml}$ of DNA.

II. EXTRACTION OF MAMMALIAN DNA

A. EXTRACTION OF HIGH MOLECULAR WEIGHT DNA. This procedure may be used to extract chromosomal DNA for genomic analysis or for the isolation of high molecular weight DNA for carrier in DNA mediated gene transfer via calcium phosphate precipitation. This is a modification of technique by Graham, et al. (1980).

1. Monolayers of cells were rinsed twice PBS and scraped into 50 ml conical centrifuge tubes.
2. The cells were spun at high speed for 4 minutes in a clinical centrifuge.
3. The pellet was rinsed twice with PBS and digested with one ml (per three confluent 100 mm dishes) of a 200 $\mu\text{g/ml}$ of proteinase K (Boehringer Mannheim) in 0.01 M Tris (pH 8.0), 0.005 M EDTA, and 0.5 percent SDS. Proteinase K is kept as a stock solution of 20 mg/ml in water and stored at -20°C .
4. After an overnight incubation at 37°C , the cell lysates were extracted once gently with phenol.
5. The aqueous upper layer was transferred to dialysis tubing with large bore pipettes, to reduce breakage of the high molecular weight DNA.

6. The DNA was extensively dialyzed against TE (pH 8.0) at 4°C. At least 6 changes of dialysis buffer was necessary to rid the DNA solution of SDS which inhibits restriction endonuclease digestion.
 7. An aliquot of the DNA sample was sheared through a 22 gauge needle several times, and the DNA concentration was spectrophotometrically determined at 260 nm. An absorbance of 1.0 is equal to 25 ug/ml of DNA in a sample of total nucleic acids (DNA and RNA) extracted from mammalian cells.
 8. The DNA is stored at 4°C.
 - *9. For Carrier DNA for calcium phosphate transfection, LTK⁻ murine cells were grown. The DNA was prepared as before, but it was aseptically sheared 10 times through a 22-gauge needle. This shearing lowers the average molecular weight of the DNA to the optimum size found for calcium phosphate transfection.
- B. Extraction of LOW MOLECULAR WEIGHT DNA. This procedure is a modification of Hirt (1967) and is used to extract viral DNA from cells. I will describe the Hirt extraction I perform for replication assays, and the procedure I use to extract SV40 DNA from CV-1 cells preparatively.

1. HIRT EXTRACTION

- a. If possible, rinse cells or monolayers of cells with PBS to wash away the serum proteins in the cell culture media.
- b. Cells are lysed with Hirt lysis buffer (30 ml of 10 percent SDS, 5 ml 1M Tris pH 8.0, 10 ml 0.5 M EDTA, 455 ml water).
- c. The solution is then made 1.0 M NaCl by the addition of one-fourth volume 5 M Na Cl. Mix well.
- d. The lysates are collected in microfuge tubes, placed at 4°C overnight, or -20°C for twenty minutes.
- e. The lysates are then spun for 10 minutes, 4°C in a microfuge.
- f. The supernatant is transferred to a new tube, and is phenol extracted, ether extracted, and then two volumes of ethanol added. The DNA is then precipitated, and resuspended in TE pH 8.0.

2. ISOLATION OF SV40 DNA

- a. Subconfluent (80 per cent) CV-1 cells were infected with approximately 10^6 - 10^7 plaque forming units of SV40 strain 776 for 3 hours.
- b. After infection, fresh DME 10 per cent FCS was added, and incubation continued for 48 hours, 37°C.
- c. Monolayers are rinsed twice with PBS.
- d. One ml of Hirt Lysis buffer was slowly added to each 100 dish.

- e. With a rubber policeman, the lysate was carefully scraped into test tubes, and was made 1 M in NaCl by the addition of 5M NaCl. During the addition and subsequent mixing, care was taken to minimize the amount of shear of the DNA.
- f. After storage at 4°C overnight, the mixture was centrifuged at 35,000 x g in a fixed angle rotor (Ti40) at 4°C.
- g. The supernatant was carefully pipetted off, and phenol extracted twice, ether extracted once, and precipitated in ethanol.
- h. The DNA pellet was suspended in 7.6 ml TE pH 8.0, and mixed with 7.8 g of cesium chloride and 0.2 ml of 10 mg/ml solution of ethidium bromide.
- i. The equilibrium centrifugation steps and purification of SV40 DNA proceeds with the steps 26-36 in Section I.C. of this appendix D.

APPENDIX E: MOLECULAR CLONING

This section is devoted to the techniques I used to clone specific DNA sequences genes for my experiments. Appendix G will contain the account of my construction of recombinant DNA plasmids pOT*-TK2 and pOT-TK5.

Molecular cloning involves screening for a particular DNA sequence and producing it in quantities appropriate for biological studies. The basic techniques for genetic engineering have already been reviewed: 1) cutting DNA with restriction endonucleases producing specific termini, 2) rejoining cleaved DNA with DNA ligase, and 3) monitoring cutting and ligation by electrophoresis. The art of genetic engineering is not the isolation of DNA fragments and their ligations, but finding that one piece of DNA among one million unique fragments.

I used the colony hybridization technique (Grunstein and Hogness, 1975) to clone the tk-gene into a SV40-recombinant plasmid. Unique DNA sequences are cloned by ligating cleaved DNA into a prokaryotic plasmid vector and transforming competent bacteria. I employed the widely popular pBR322 (and its derivative pML6, Lusky and Botchan, 1981) plasmid vector with the E. coli host HB101. Plasmid pBR322 contains two genes that confer ampicillin and tetracycline resistance to E. coli., an origin of replication, and several unique restriction endonuclease sites (Bolivar, et al., 1977). PBR322 plasmid DNA is restricted with an appropriate endonuclease and ligated to a mixture of similarly restricted foreign DNA. The ligation mixture is introduced into bacteria that are antibiotic sensitive by a procedure

called bacterial transformation. Only the cells that have these plasmids can survive on media containing antibiotics. Replicas of the colony laden plates are made with nitrocellulose filters. The bacteria are lysed in situ, their DNA bonded to the filter and hybridized to radioactively tagged nucleic acids. After hybridization and wash, X-ray film is exposed to the radioactive filters. The resulting autoradiogram locates the recombinant clone of interest. Figure A.2 depicts a representative autoradiogram of a probed filter.

I. PRODUCTION OF TRANSFORMATION COMPETENT HB101 CELLS:

A. Materials

1. Glycerol - autoclaved
2. 0.1 M CaCl_2 - autoclaved
3. 0.1 M MgCl_2 - autoclaved
4. LB media - 10 g NaCl, 10 g Bacto-Tryptone, 5 g Yeast Extract, 2 ml 1 N NaOH, 1 liter water, autoclave
5. Eppendorf microfuge tubes - autoclaved
6. Autoclaved 400 ml centrifuge bottles for GS3 rotor.

B. Protocol

1. Inoculate a fresh overnight culture of HB101 in one liter of LB media. Incubate with shaking at 37°C.
2. When Absorbance at 600 nm is 0.7 take incubation flask and plunge it into a large ice-water bath to quickly bring temperature to 0°C.

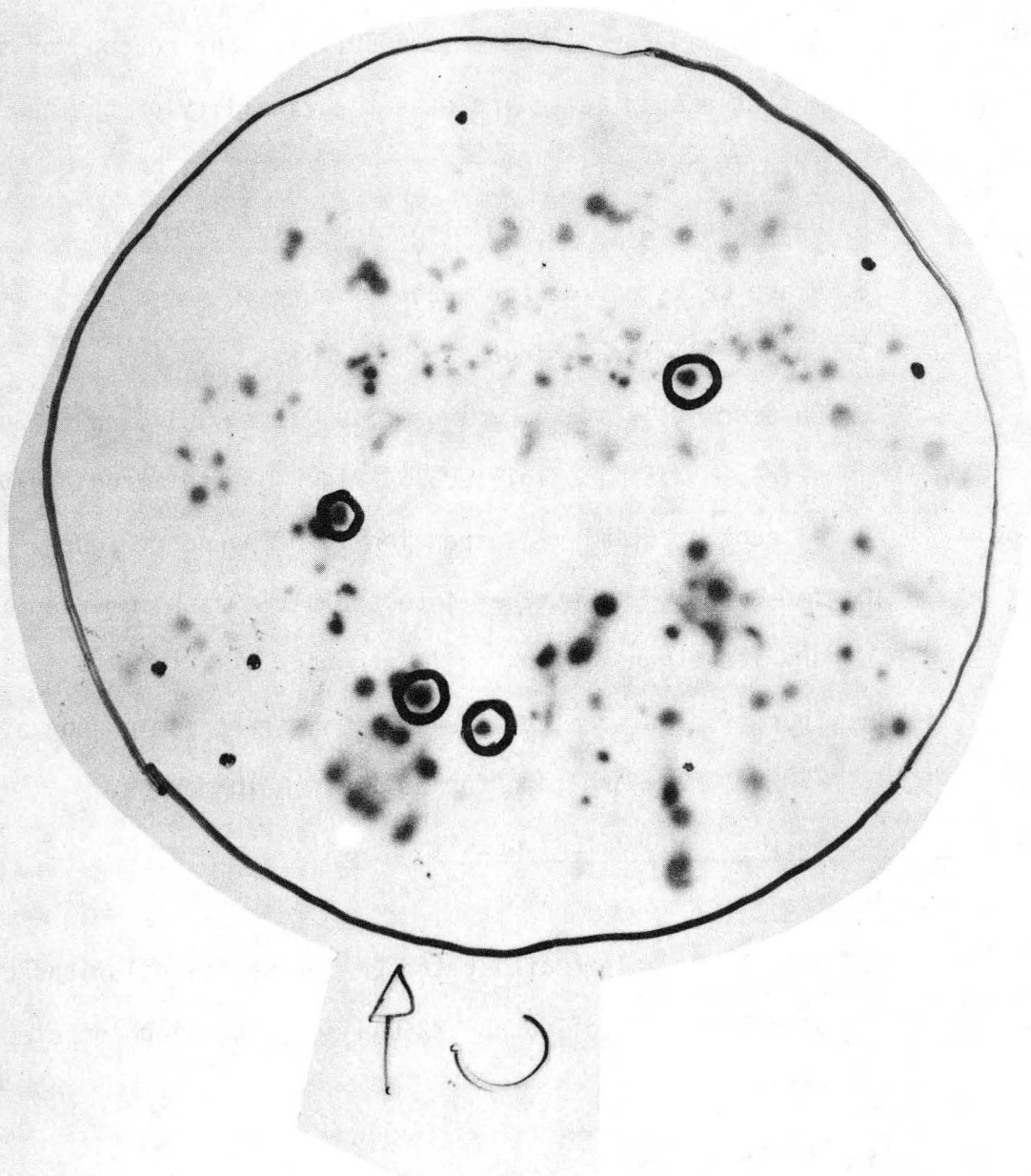


Figure A.2. Autoradiogram from a typical colony-hybridization experiment.

XBB 844-2991

3. Spin cells for 1 minute at 5000 rpm.
4. Resuspend cells in 250 ml of ice-cold 0.1 M $MgCl_2$.
Spin cells for 1 minute at 5000 rpm. The reason for such a gentle spin is to increase the viability of the now fragile bacterial cells.
5. Resuspend cells in 250 ml of ice-cold 0.1 M $CaCl_2$.
6. Place cells on ice for twenty minutes.
7. Spin cells for 1 minute at 5000 rpm.
8. Suspend cells in 42.5 ml of cold 0.1 M $CaCl_2$ and then swirl in 7.5 ml of glycerol. Place on ice immediately.
9. Aliquot 0.250 ml of suspension into Eppendorf tubes.
10. Immediately place tubes into a plastic rack immersed in a dry ice-ethanol bath for 10 minutes.
11. Store frozen competent cells in an ice-cream carton at $-70^{\circ}C$. They are good for at least 8-10 months.

II. TRANSFORMATION OF COMPETENT CELLS

A. Materials

1. DNA: 10 μ l volume of ligated DNA or serial dilutions of plasmid super-coiled pBR322 DNA for titrating competent cells.
2. Competent HB101 cells
3. LB media
4. LB-ampicillin plates (100 μ g/ml amp)
 - a. for one liter volume: 10 g NaCl, 10 g Bacto-tryptone, 5 g Yeast Extract, 15 g bacto-agar.

- b. autoclave liquid cycle for 20 minutes.
- c. Before pouring, add 4 ml of a 25 mg/ml ampicillin sterile stock solution.

B. Protocol

1. Place frozen tube of competent bacteria in ice-bucket.
2. Place 10 μ l of DNA solution in a sterile 14 ml-test tube, place test-tube on ice.
3. Add 0.1 ml of thawed cell suspension to the 10 μ l of DNA. Incubate on ice for 10-20 minutes.
4. Heat-shock culture by incubating at 37°C for 3-5 minutes.
5. Incubate on ice for 10-20 minutes.
6. Add 2.0 ml of LB media without antibiotics, incubate on shaker at 37°C for 45-60 minutes.
7. Pipette 0.10-0.20 ml of bacterial suspension on agar plates, spread with sterilized glass spreader.
8. Incubate for 16 hours at 37°C with bottom-side up.
9. A population of efficient competent cells will titer to a transformation frequency of 10^6 - 10^7 colonies per microgram of supercoiled pBR322 DNA.
10. For colony hybridization, there should be approximately 200-300 well spread out colonies per plate. If there are too many or too few, replate at appropriate concentration.

III. COLONY HYBRIDIZATION

A. MATERIALS

1. Agar plates with 200-300 bacterial colonies.
2. Nitrocellulose filters (Millipore, HATF 08225, 0.45 μ m pore size).
3. Boilable cooking pouches (Sears Roe., No. 34 C6546 (8 x 12"), No. 34 C6558 (8x9")) and pouch sealer.
4. Nick-translated 32 P-labeled DNA probe.
5. Whatman 3MM chromatography paper.
6. Gel Soak I and Gel Soak II Solutions
7. Hybridization solution - 100 ml
 - a. 30 ml 20X SSC
 - b. 5 ml 10 per cent SDS
 - c. 20 ml 50X Denhardtts
 - d. 45 ml distilled water.
8. Filter Wash solution: 2X SSC and 0.1 percent SDS (pre-warmed to 68°C).
9. Kodak XAR-5 film and Dupont Cronex intensifying screen.
10. Wolfe X-ray exposure cassette.

B. Protocol

1. With a No. 2-pencil, lightly label nitrocellulose filter.
2. With two blunt-tip filter-tweezers, place labeled nitrocellulose filter on the agar plate with colonies; let filter get wet from the agar by itself, do not press.

3. With a needle, place alignment holes into the filter and agar plate. These holes will be needed later to orient the autoradiogram on the master plate with colonies.
4. On a flat surface, tape down a piece of plastic Saran wrap. Place, evenly spaced, 0.75 ml of gel soak I.
5. Lift filter carefully off each agar dish and place colony-side up on the drop of gel soak I. The master plate will probably be left with only impressions of the original colonies. Place these master plates in a 37°C incubator for 5 hours and then store them at 4°C.
6. After about 30 seconds, lift the filters off Saran wrap and place them on clean, dry Whatman 3M paper. Let dry for approximately one minute.
7. Transfer filters a second time to Saran wrap with 0.75 ml Gel Soak I, 30 seconds, transfer to dry Whatman 3MM paper.
8. Transfer filters to Saran wrap with 0.75 ml Gel Soak II, thirty seconds, transfer to Whatman.
9. Repeat step 8.
10. Dip filters in 3M NaCl three times, then dry filters on Whatman. Transfer to dry Whatman, and let air dry.
11. Cut Whatman paper into squares a little larger than nitrocellulose filters. Fold each one in half to make a crease.

12. Form a "sandwich" column of Whatman paper, filter, Whatman paper, filter, etc., with each Whatman oriented to give the crease at 90 degrees off the former. This will keep the filters from sticking to the Whatman when baking. Wrap Whatman and filter assembly in aluminum foil.
13. Bake in vacuum oven for 2 hours at 80°C. Pull out and let cool.
14. Wearing plastic gloves, carefully place all the filters (without the Whatman paper) one on top of the other in a boiling bag. Seal off excess space, leaving about a one centimeter border on three sides.
15. Add 15–20 ml of hybridization fluid. Seal bag, leaving a 3 centimeter margin, and expel all air-bubbles before sealing.
16. Incubate at 68°C for 4–6 hrs—this is pre-hybridization.
17. Boil for 5 minutes nick-translated probe, place immediately on ice—this step denatures the probe.
18. With a syringe and a 22-gauge needle, inject probe into the pre-hybridized mixture with filters. Seal corner where injection made. Dispose of needle and syringe properly.
19. Incubate at 68°C with shaking, preferably in a water bath, for 12–16 hours.
20. Pre-warm, over-night, the Wash solution at 68°C.

21. After hybridization, cut corner of hybridization bag, and carefully pour out radioactively labeled hybridization solution into appropriate containment vessel.
22. Put filters immediately in a plastic box (with a cover) with wash solution. Rinse twice with pre-warmed wash solution.
23. Add 0.5-1.0 liter of pre-warmed wash solution, close lid and incubate for 30 minutes with shaking in a 68°C water bath.
24. Rinse and repeat step 24.
25. Rinse filters in 2X SSC and air dry on Whatman paper.
26. Mount dried filters on Whatman paper with tape, cover mounted Whatman paper with Saran wrap.
27. Expose X-ray film (with or without screen) to mounted filters.
28. Develop autoradiogram.
29. Using felt-tip pen, outline filters on Autoradiogram and place marks on alignment pin-holes, using mounted filters.
30. Using marked autoradiogram, pick isolated clones that line-up with exposed colony record.
31. Grow selected clones in LB media with ampicillin, and analyze as described in Appendix D.

APPENDIX F: CONSTRUCTION OF PLASMIDS pOT*-TK2 and pOT-TK5

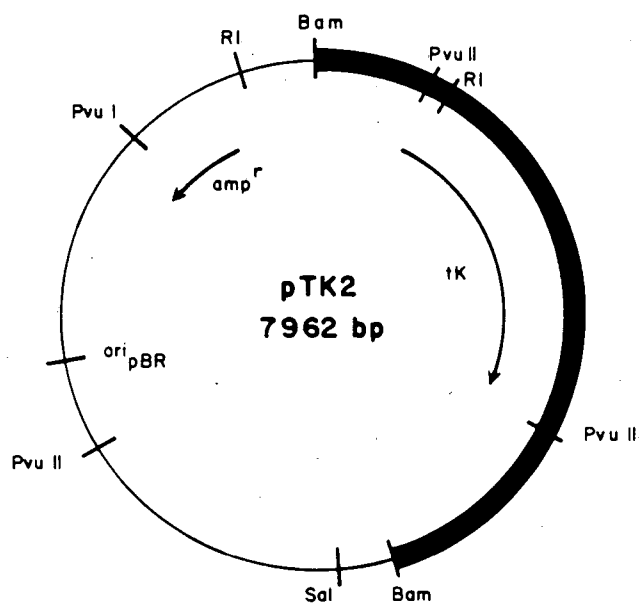
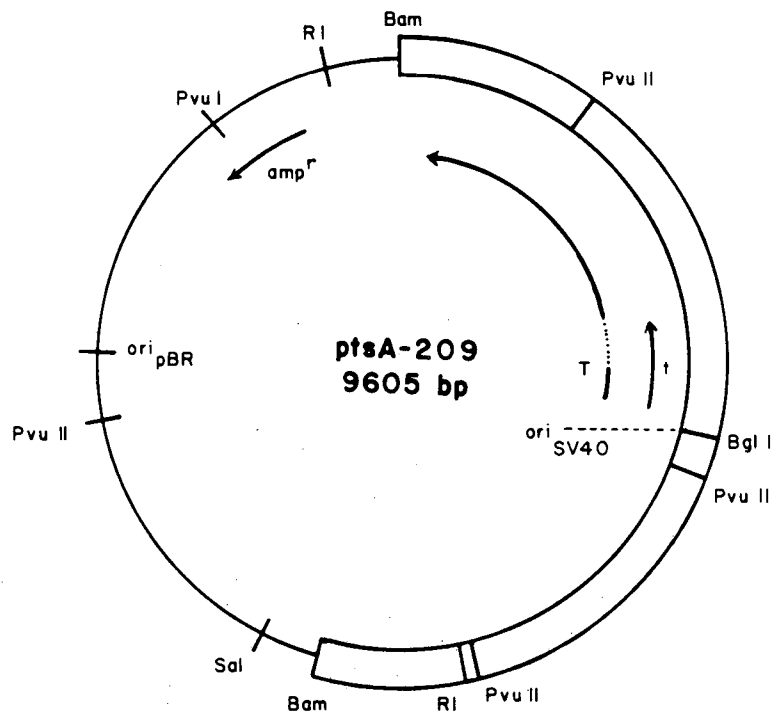
In this section I will describe the construction of plasmids pOT*-TK2, pOT-TK5, and their precursor plasmids. I assembled all the genetic engineering techniques used for these constructions in Appendices B, C, D, and E.

Plasmid pTK2. This plasmid was a gift from Michael Botchan. The 3.4 kbp Bam HI tk-gene fragment from Herpes Simplex Virus Type I was cloned into the unique Bam HI site of pBR322. The orientation was confirmed by digestion with Eco RI producing the predicted 1.06 and 6.9 kbp fragments (Figure A.3).

Plasmid pJY1. This plasmid was a gift from Monika Lusky. This plasmid contains SV40 viral DNA cleaved at its unique Bam HI site cloned into the unique Bam HI site of pBR322. This plasmid cannot replicate in CV-1 or COS-7 cells.

Plasmid PJYM. This plasmid was a gift from Monika Lusky. This plasmid is a deletion derivative of pJY1. Detailed restriction analysis and DNA sequence analysis reveals that the specific deletion lies within the pBR322 DNA spanning the region between nucleotide positions 1092 and 2485 (Lusky and Botchan, 1981). PJYM will replicate in CV-1 and COS-7 cells.

Plasmid pML. This plasmid is the result of deleting the SV40 Bam HI fragment from pJYM. PML is 2.97 kbp and still contains an intact ampicillin resistance gene and unique restriction sites Bam HI, Eco RI, Hind III, Sal I.



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Figure A.3. Physical Maps of Plasmids ptsA-209 and pTK2.

Plasmid ptsA-209. This plasmid was constructed by inserting Bam HI cleaved tsA-209 DNA (a gift from Michael Botchan) into the unique Bam HI site of pBR322 (Figure A.3). Orientation was confirmed by digestion with Eco RI which produced the predicted 4.87 and 4.74 kbp fragments.

Plasmid pD19. This plasmid was constructed to exchange the 1446 bp PVU II fragment in the SV40 late region in ptsA-209 with the Pvu III 2025 bp tk-gene fragment from pTK2.

FRAGMENT ISOLATION:

1. 50 μ g of PTK2 DNA was cleaved to completion with 50 units of Pvu II, and electrophoresed. The 2.0 kbp fragment was isolated by glass beads (Appendix C.IV).
2. 50 μ g of ptsa-209 was cut for 1 hour with 10 units of Pvu II and electrophoresed on a 1 per cent agarose gel. This partial digestion produces fragments of different sizes. The 8.16 Pvu II fragment was isolated by glass beads.

Clone isolation: The isolated fragments were ligated and the ligation mixture was used to transform HB101.

Colony-hybridization analysis was performed on bacterial colonies, using nick-translated Pvu II-tk fragment as probe.

pD19 confirmation: Restriction analysis that verified pD19 construction (Figure A.4).

1. Pvu II digestion verified exchange of 1446 bp SV40 fragment for 2025 k bp fragment of herpes tk-gene.
2. Orientation verified with digestion by Eco RI which cuts the TK-fragment assymmetrically.

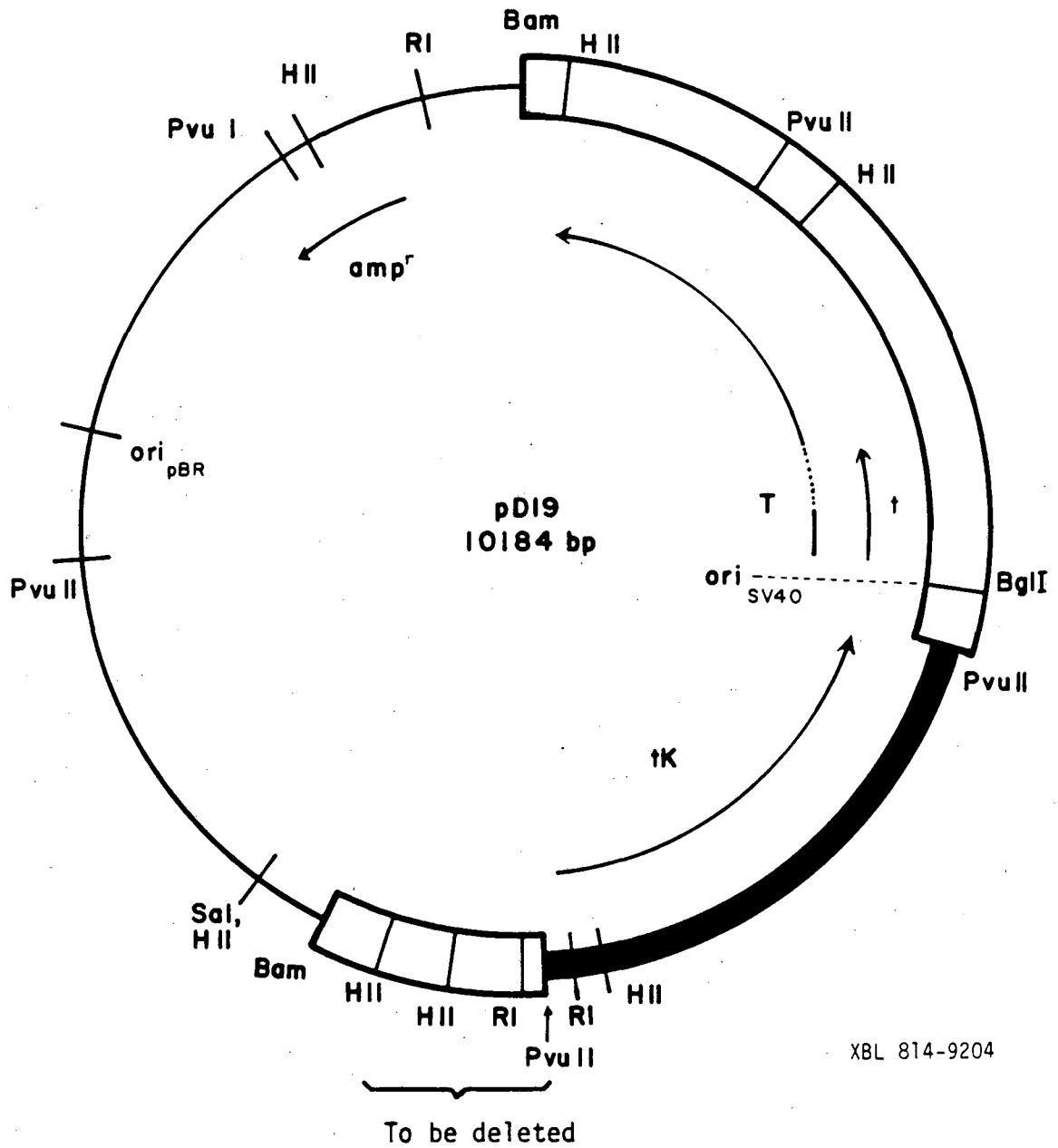


Figure A.4. Physical map of plasmid pD19.

Plasmid pD19 produces two Bam HI fragments: the 4362 bp pBR322 DNA and the 5822 bp recombinant SV40-tk gene DNA. SV40 can only package 3 per cent more DNA ($5243 \times 1.03 = 5400$ bp), therefore, plasmid pD19 must be deleted by at least 422 (5822-5400) base pairs of DNA. I concluded that the DNA between the Pvu II junction site at the 5'-end of the Tk-gene and the Hind II site near the Bam HI junction of SV40 and pBR322 (Figure A.4) would delete 581 base pairs of DNA. Since Hind II and Pvu II digested DNA blunt ended termini. Unfortunately performing simultaneous Hind II and Pvu II partial digestions simultaneously and finding a 9603 bp fragment appeared fruitless. It would be far easier to perform a Hind II partial digestion in one reaction and a Pvu II partial hydrolysis in another reaction. The appropriate fragments could then be isolated and digested to completion with the enzyme Pvu I which cuts at a unique site in the ampicillin resistance gene. But even this approach was made difficult by the fact that pD19 contains 7 Hind II sites. I decided instead to delete the Hind II site that resides at the same recognition sequence for Sal I. Hind II cuts at recognition sequences GTTAAC or GTCGAC. GTCGAC is also the recognition sequence for Sal I. By performing a filling-in reaction with the Klenow fragment of DNA polymerase I (Appendix C.V) upon pD19 DNA digested with SALI, I would eliminate the recognition sequence for the Sal site and the concomitant Hind II site. Ultimately, I would be changing the DNA sequence GTCGAC to GTCGATCGAC by the effective insertion of TCGA. The resultant plasmid pD19-deltaSAL would contain only six Hind II sites,

but there would be no Hind II site in the PBR322 DNA between Pvu I and the Hind II site near the Bam HI junction site of SV40 and pBR322. PD19(delta Sal) would then be cut to completion with both Hind II and Pvu I and ligated to the fragment isolated from a partial Pvu II and Pvu I complete digest of pD19.

Plasmid pD19 (delta SAL). Plasmid pD19 was cut to completion with Sal I. The cleaved plasmid was filled in with the action of the Klenow fragment of Dna polymerase I (Appendix C.V). One microgram of filled in DNA was transformed into HB101, and clones were isolated. Verification of the plasmid was confirmed by Hind II digest, and lack of Sal I site.

Plasmid pOT*-TK1. This plasmid will now contain the appropriately tailored SV40-tk recombinant.

Fragment isolation:

1. Plasmid pD19 (deltaSAL) was cut to completion with Hind II and Pvu I. The 3.36 kbp fragment was isolated with glass beads.
2. Plasmid pD19 was partially cut with Pvu II and to completion with Pvu I. The 3.98 kbp fragment was isolated with glass beads.

Clone Isolation: Fragments were mixed and ligated. The ligation mixture was transformed into HB101 cells. Since Pvu I cuts directly into the ampicillin resistance gene, correct ligation reconstitutes the gene. Only the desired construction should result--no need for colony hybridization.

POT*TK1 confirmation: Restriction analysis verified this construction. Bam HI digest confirmed that the SV40-tk fragment was the same size of Bam HI digested SV40. Pvu II digest confirmed junction (pOT*-TK1 contains 3 and not 4 Pvu II sites).

Plasmid pOT*-TK2. I cloned the Bam HI SV40-TK fragment into pML to perform direct replication studies of the recombinant DNA plasmid without restriction isolation of the recombinant DNA.

Fragment isolation:

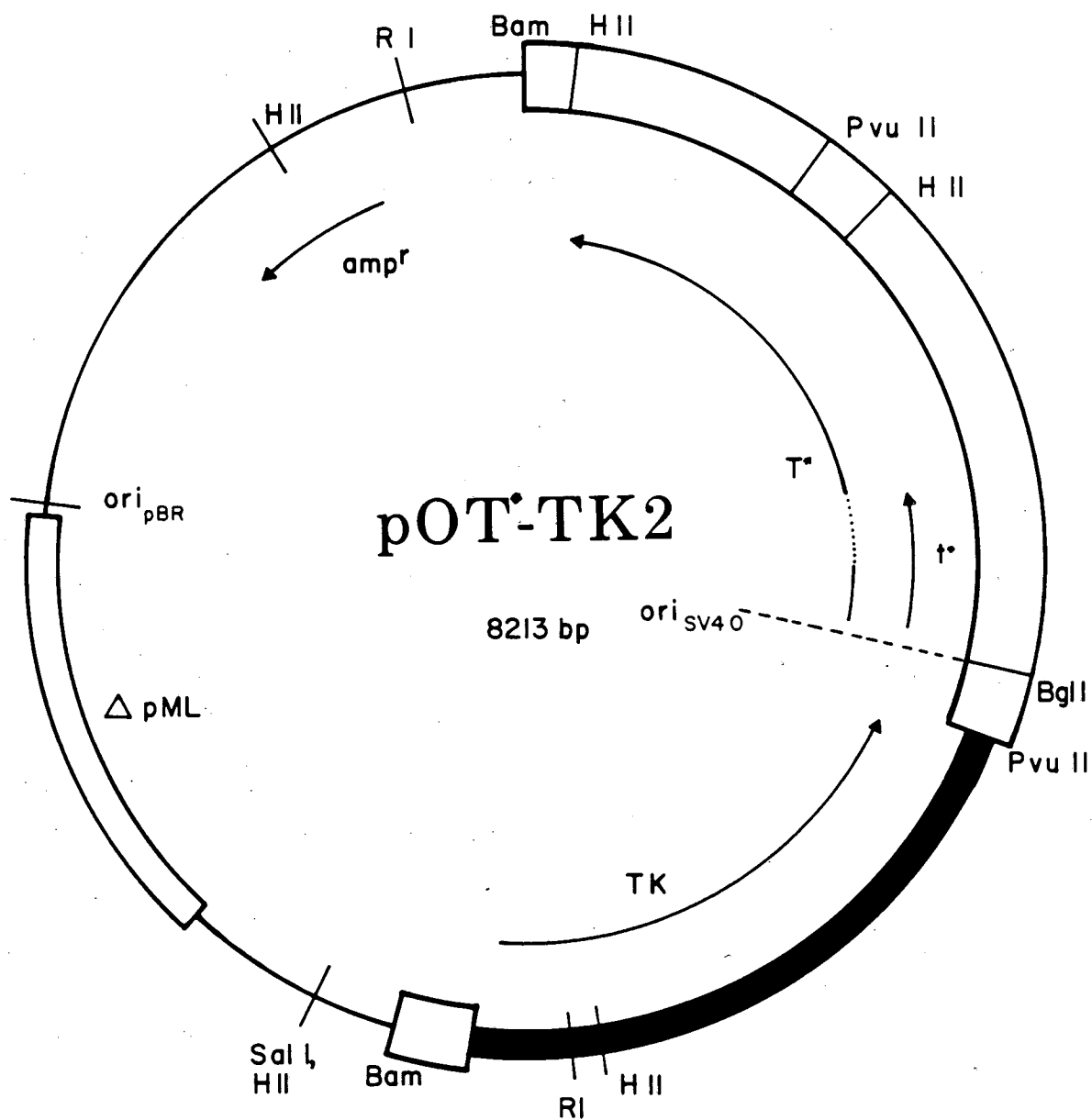
1. Plasmid pOT*-TK1 was cut to completion with Bam HI. The 5241 bp fragment was isolated by glass beads.
2. Plasmid pML was cut to completion with Bam HI.

Clone isolation: HB101 cells were transformed with the ligation mixture. Colony-hybridization analysis was performed on the bacterial colonies, using nick-translated SV40 DNA as probe.

POT*-TK2 confirmation: Restriction analysis verified pOT*-TK2 construction (Figure A.5):

1. Bam HI digestion verified that Bam HI SV40-tk fragment was cloned into pML.
2. Orientation was verified by Eco RI digestion. Eco RI cuts the SV40-tk recombinant assymmetrically.

Plasmid pOT-TK5. I wanted an SV40-TK recombinant that contained the wild-type A-gene. The tsA-209 mutation maps to the Hind II/Hind III "I" fragment by marker rescue (Lai and Nathans, 1975). I decided to



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Figure A.5. Physical map of plasmid pOT*-TK2.

exchange the 2007 bp Pvu II fragment of wild-type SV40 with the 2007 bp Pvu II fragment of pOT*-TK2. Pvu II actually cuts 30 bp within the I-fragment of the Hind II/Hind III digest, but I could test biologically by replication assay for the localization of the tsA-209 mutation in this construction.

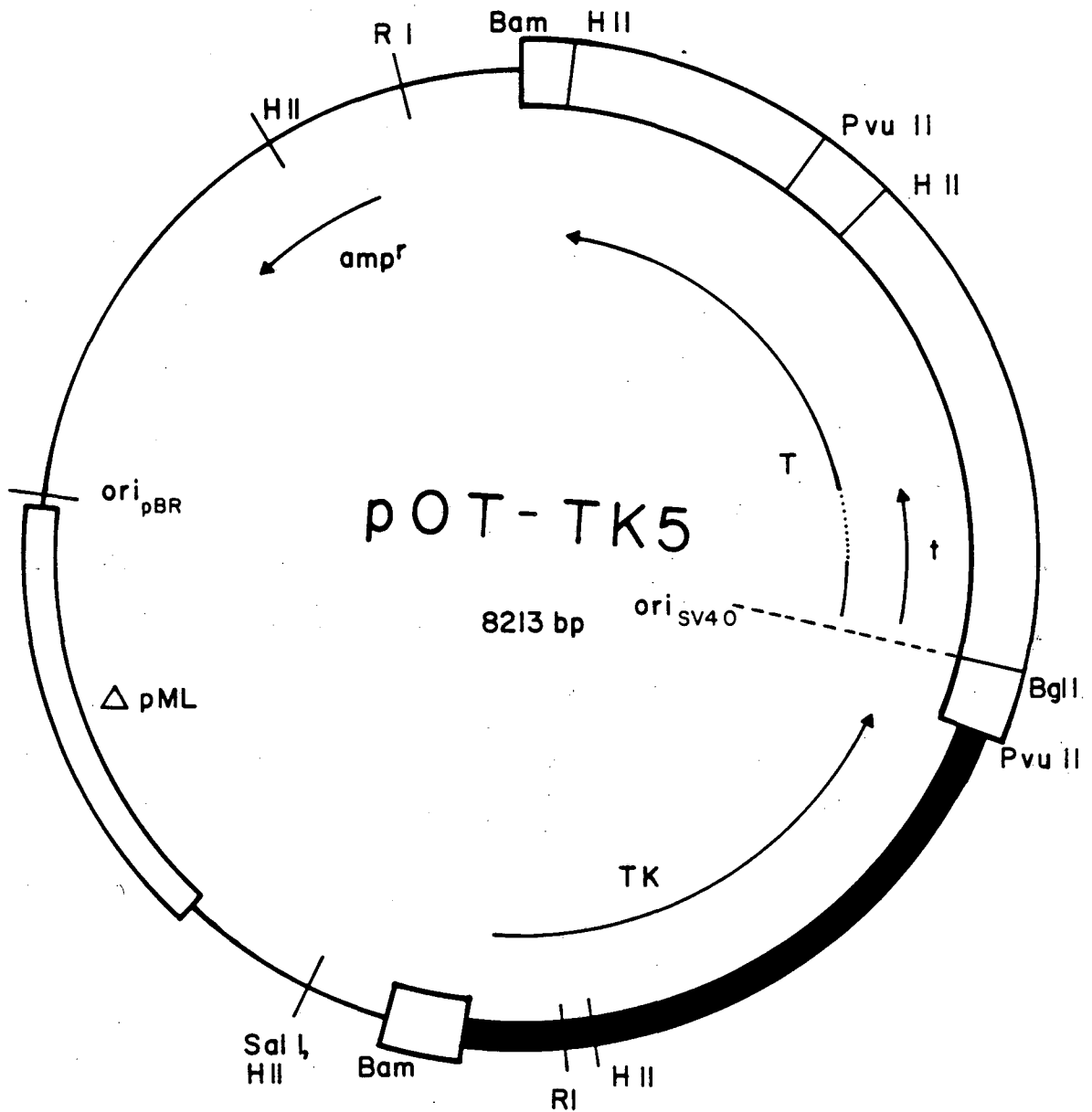
Fragment isolation:

1. Plasmid pOT*-TK2 was cut to completion with Pvu II. The 7596 bp fragment was isolated by glass beads.
2. Wild-type SV40 viral DNA was cut to completion with Pvu II. The 2007 bp fragment was isolated by glass beads.

Clone Isolation: The ligation mixture was transformed into HB101 cells. Colony-hybridization analysis was performed on the bacterial colonies, using nick-translated 2007 bp Pvu II fragment from SV40 viral DNA as probe.

POT-TK5 confirmation:

1. Restriction Analysis: Hind II digest of pOT*-TK2 and pOT-TK5 were identical as predicted (Figure A.6).
2. Biological verification: Replication assay. A Hpa II digest of either pOT*-TK2 or pOT-TK5 will liberate a complete SV40 early region, coding for the T-antigens, and an intact SV40 origin. Hpa II digests of both plasmids were transfected into CV-1 cells at 37°C. The Hpa II digest of pOT-TK5 replicated at both 33 and 39°C. The Hpa II digest of pOT*-TK2 replicated at 33°C but not at the non-permissive temperature of 39°C. Later studies also showed that A-gene



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Figure A.6. Physical map of plasmid pOT-TK5.

transformants of pOT-TK5 transfected Rat-2 cells did not show any temperature sensitive modulation of the transformed phenotype.

APPENDIX G: SOUTHERN TRANSFER AND HYBRIDIZATION

Specific DNA sequences can be located within DNA fragments by the application of transfer techniques described by Southern (1975). DNA fragments that have been separated according to size by electrophoresis through agarose gels are denatured in situ, transferred to nitrocellulose filters by capillary action, and immobilized on the filters. The relative positions of the fragments during electrophoresis are preserved during the transfer to the nitrocellulose filter. The DNA bonded to the filter is hybridized with either ^{32}P -labeled DNA or RNA, and autoradiography of the nitrocellulose filter locates the bands of DNA that are complementary to the probe. This technique is extremely sensitive, by analyzing 15 μg of mammalian cellular DNA it is possible to recognize as few as 100 base pairs of foreign DNA per diploid equivalent of cellular DNA. I modified the method of Southern to 1) analyze the structure of integrated plasmid pTK2 DNA integrated into Rat-2 cells, 2) assay replication of recombinant SV40 plasmids in simian cells, and 3) quantitate the amount of DNA that entered the nuclei in transfected Rat-2 cells.

I. MATERIALS AND EQUIPMENT

A. MATERIALS.

1. Nitrocellulose filter membrane (Schleicher and Schuell BA 85 or Millipore HAHY). Use plastic gloves and Millipore forceps to handle the nitrocellulose.

2. Boilable cooking pouches (Sears Roe., No. 34 C6546 (8"x12"), No. 34 C6558 (8"x9")) and pouch sealer.
3. Nick-translated 32P-labeled DNA probe.
4. Whatman 3MM chromatography paper.
5. Gel Soak I and Gel Soak II.
6. 20 X SSC.
7. Hybridization solution - 100 ml
 - a. 30 ml 20X SSC
 - b. 5 ml 10 per cent SDS
 - c. 20 ml 50X Denhardts solution.
 - d. 45 ml distilled water.
 - e. Sterilize hybridization solution through Nalgene filter.
8. Filter Wash solution: 2 x SSC and 0.1 percent SDS.
9. Kodak XAR-5 film and Dupont Cronex intensifying screen.
10. Glass borosilicate disposable 10 ml pipettes.
11. Syringe fitted with 22-gauge needle.
12. Millipore filter forceps.
13. Distilled Water.
14. 10 mg/ml ethidium bromide solution.
15. Plastic wrap (Saran).
16. Wolfe X-ray exposure cassette.

B. Equipment

1. Vacuum drying oven, 80°C.
2. Self-filling water bath, 68°C, with shaker.

3. Boiling rack for Eppendorf tubes.
4. Tight-sealing plastic boxes for incubating filters.
5. Pyrex baking dishes, 4.
6. Electrophoresis equipment.
7. Glass plates that fit into Pyrex baking dishes.
8. Radioactive Waste (dry and liquid) containment vessels.
9. Geiger counter.
10. X-ray developing equipment and dark room.
11. Ultraviolet box.

II. PROTOCOL

A. PREPARATION OF AGAROSE GEL.

1. Pour an agarose gel (0.75 to 1.1 per cent) that is at least 1 cm thick. Pick a comb whose teeth will provide a gel slot that has 2-3 times the volume of the DNA sample to be loaded. This precaution eliminates DNA streaming out of the gel at the top of the slot, and gives a cleaner transfer.
2. Load DNA samples and appropriate DNA markers and electrophorese for desired time and voltage. For genomic analysis, I find that better resolution is acquired when electrophoresis proceeds overnight at low voltage.
3. Stain gel with a 1:10,000 dilution of ethidium bromide stock solution in water. Stain for at least 30 minutes.

4. UV irradiated gel for five minutes on UV gel box. This breaks up the large fragments, and makes it easier for transfer.
5. Optional step: When blotting replication assay, the covalently closed supercoiled DNA transfer rather inefficiently. Soaking gel for 15 minutes, with gentle shaking in 0.25 M HCl hydrolyzes the DNA partially by acid depurination. Do not over soak, otherwise the fragments will be too small for efficient transfer.
6. Neutralize the gel by soaking in several volumes of Gel Soak for 30 minutes. Be sure the gel is bottom-down and top-up. The gel can be easily submerged by placing three 10 ml borosilicate pipettes gently on top of gel.
7. Rinse gel with several changes of tap water, being careful not to tear gel, wear gloves.
8. Soak Gel in several volumes of Gel Soak II for 30 minutes. The gel is ready for transfer, by this time you should have prepared the nitrocellulose filter and built the transfer apparatus.

B. NITROCELLULOSE FILTER PREPARATION.

1. My gels are usually 15 cm x 15 cm. Cut a piece of nitrocellulose that is 15 x 15 cm. Wear gloves. I find that using the glass plate as a template, I can cut a filter with a clean razor blade. Always keep the filter between the protective non-stick sheets provided by the vendor.

2. Cut 3 pieces of Whatman 3MM paper the same size as the nitrocellulose filter. Wear Gloves, and keep paper clean.
3. Wet nitrocellulose filter in distilled water in a clean Pyrex baking dish. Nitrocellulose that has been touched by greasy fingers will never wet.
4. Place wetted filter in a baking dish of 20 X SSC, until ready to build apparatus.

C. PREPARATION OF TRANSFER APPARATUS.

1. Cut two pieces of Whatman 3MM paper that are 15 cm x 40 cm.
2. Place in a clean pyrex baking dish a disposable pipette tip rack (or anything plastic that is about 3 cm thick).
3. Place a glass plate that is at least the same width of the agarose gel and filter.
4. Fill Pyrex dish with 20XSSC, to a height of 2 cm.
5. Wet the two long Whatman papers in 20XSSC and place them on the glass plate so that both free ends are dipping into the 20X SSC. These papers will act as the wick to bring the 20X SSC to the gel.
6. The gel apparatus is now ready to be loaded.

D. SOUTHERN TRANSFER

1. Place the gel (after soaking in Gel Soak II) on the Whatman paper wick, with the bottom of the gel facing up. We purposely loaded the gel so that the DNA samples

are nearer the bottom of the gel. By placing the gel upside down, the transfer of fragments will be more efficient.

2. If there are any exposed Whatman surfaces on the glass plate, cover them with either cut X-ray film, or Parafilm. The films will act as barriers for the short-circuiting of the 20 x SSC.
3. Carefully transfer the nitrocellulose filter soaking in 20 X SSC and carefully place it on top of the gel. Be sure to wipe away any bubbles that get sandwiched between the gel and the nitrocellulose filter.
4. Dip one of the pre-cut Whatman squares in 20 X SSC and place on top of Nitrocellulose filter. Be sure to press away any bubbles formed. Repeat with the other two Whatman precut squares.
5. Place a stack of (10-12 cm) of clean paper towels on top of the Whatman squares. If you have been careful in planning, the samples to be transferred should be loaded in a total width equal to that of the paper towels. If not, you may overlap the towels for 4 cm, and then add a 8 cm stack.
6. Place a glass plate on top of the paper towel stack, and place a light weight (150-300 grams) on top of the plate.
7. Allow transfer of DNA to proceed for about 12-24 hours. As the towels become wet, they should be replaced. The

rate of transfer of DNA depends on the porosity of gel and the size of the DNA fragments. Small fragments of DNA (less than 1000 bp) transfer from an 0.8 percent agarose gel within three hours while transfer of DNA greater than 12 kbp takes 15 hours or more.

8. Carefully remove the paper towels and the Whatman squares.
9. With a hyperdermic needle, mark the position of the gel on the nitrocellulose filter with desired number of holes at the four corners. You should try to make marks that will allow exact alignment if needed.
10. With a plastic squirt bottle, rinse the gel side surface of the nitrocellulose filter with 2 X SSC to rid the filter of any agarose particles.
11. Allow filter to air dry on 3MM paper at room temperature.
12. Place the dried filter between two sheets of 3MM paper. Bake for 2 hours at 80°C in a vacuum oven.
13. I try to use the filter immediately for hybridization.

D. HYBRIDIZATION AND AUTORADIOGRAPHY.

1. The baked nitrocellulose filter is brittle, so carefully slide it in the boiling pouch. I use the 3MM paper as a rigid surface to slide the filter in, then carefully pull the 3MM paper out. Always wear gloves.

2. Add 15 - 20 ml of hybridization solution to pouch, so that a noticeable 1.5 cm bulge of fluid resides at the bottom of the pouch when held up.
3. After getting rid of all bubbles, seal bag. Always make two seals so that solution can not leak out. Leave at least 6 cm work space above the filter at the top of the boiling pouch for probe introduction.
4. Place in plastic box filled with water, and incubate with shaking in a 68°C shaker-water bath. Pre-hybridize for at least 6 hours.
5. Prepare probe for hybridization by boiling 1.00 ml of a freshly prepared nick-translated DNA (1.0 ug) in an Eppendorf tube held in a boiling rack. Boil for five minutes, then immediately place on ice. The rapid temperature change keeps the denatured DNA fragments from rehybridizing with themselves before injection.
6. Wearing gloves and in a radiation containment area, inject the 1.00 ml probe at one of the top corners of the boiling pouch with a syringe fitted with a 22-gauge needle.
7. Seal the corner of bag after expelling any air bubbles. Injection is the most efficient means of introducing probe without loss of hybridization fluid and without adding air bubbles.

8. Hybridize for 16 hours in shaker bath as before, 68°C.
9. After hybridization, cut corner of boiling pouch, and pour radioactive hybridization solution into appropriate liquid radiation waste vessel.
10. Fill a plastic, sealable box with 300 ml of pre-warmed (68°C, warmed overnight in a 68°C incubator, 2 liters) filter wash solution.
11. Carefully place nitrocellulose filter in the rinse vessel, and pour off radioactive rinse.
12. Fill vessel immediately (do not let filter dry) with one liter of pre-warmed wash solution, and seal box.
13. Place in shaker bath, incubate 68°C, 30 minutes with shaking.
14. Repeat steps 11, 12 and 13.
15. After the second wash, depending on the expected signal, the filter should not have any not specific counts as crudely measured with a Geiger counter.
16. Rinse filter with 2 X SSC at room temperature, and let the filter air dry on 3MM paper.
17. Mount dry filter on 3MM paper, and wrap with Saran plastic wrap.
18. In dark room, place 3MM paper with mounted filter, X-ray film, and intensifying screen (smooth surface up) in Wolfe exposure cassette.

19. Expose at -70°C for appropriate time, then develop X-ray film. For genomics an exposure of 14 days is sometimes necessary while for replication assays only 3-4 days are needed.

APPENDIX H: DNA MEDIATED GENE TRANSFER TECHNIQUES

I have used DEAE dextran and calcium phosphate precipitation to transfect DNA into mammalian cells.

I. DEAE DEXTRAN. This transfection technique works the best with cell lines CV-1 and COS-7. It is easier than the calcium phosphate precipitation--which tends to be toxic to the simian cells. I used a modification of McCutchan and Pagano (1968).

A. MATERIALS

1. Tris buffered saline (TBS)
2. Phosphate buffered saline (PBS)
3. DEAE-Dextran (Sigma D9885; Molecular Weight 500,000 in a 10 mg/ml stock solution in TBS. I filter sterilized the stock solution with no loss of activity. Store at 4°C.

B. PROTOCOL

1. Prepare sub-confluent CV-1 or COS-7 cells in 60 mm dishes.
2. Dilute DEAE-Dextran stock to 0.5 mg/ml with TBS.
3. Add DNA to concentrations: 50 ng/ml for replication assays or 5 μ g/ml for plaque assays.
4. Rinse cells once with PBS, then once with TBS.
5. ADD 0.20 ml of DNA solution. Incubate for 30 minutes at 37°C.
6. Rinse once with TBS, and then once with PBS.
7. Add normal growth media.

8. Plaque Assays

- a. Prepare 2X DME solution from powder filter sterilize.
- b. Prepare a 0.8 per cent Noble agar (Difco) solution by autoclaving.
- c. Equilibrate both solutions at 50°C, mix equal volumes, and add 5 ml to aspirated dishes.
- d. Let agar harden at room temperature, 10 minutes.
- e. Incubate at desired temperature.
- f. Add 3 ml agar media every 4 days.
- g. On 15th day add 2ml agar with 1:100 dilution of neutral red solution (Gibco). Incubate overnight at 37°C.

II. CALCIUM PHOSPHATE PRECIPITATION

A. MATERIALS

1. 2X HBS (Hepes Buffered Saline)

- a. Dissolve 3.7 g KCl, 10.0 g D-Glucose, and 1.0g Na_2HPO_4 in a total volume of 50 ml water.
- b. In a plastic beaker, add 1 ml of the salt solution, 1.0 g Hepes (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid, Sigma), and 1.0 g NaCl. Bring up to a volume of 85 ml.
- c. pH to 7.05-7.10 with 1 N NaOH, adjust volume to 100ml and sterilize by filtration.

2. 2.5M CaCl_2 , sterilize by filtration.

3. Water, pH 7.05-7.10, sterilize by filtration.

4. Store all reagents in plastic conical 50 ml test tubes, at -20°C .
5. For precipitation, use 4ml or 14 ml clear polypropylene sterile test tubes. These tubes make precipitate easier to visualize.

B. PROTOCOL

1. Prepare sub-confluent or confluent cell cultures in 100 mm dishes, with 10 ml of culture media.
2. For my experiments, I generally prepare a large calcium phosphate cocktail mixture to transfect many plates simultaneously. I will describe the amount procedure for a total of 1 ml of solution. Scale up the amounts as necessary, allow for appropriate amount for transfer errors.
3. For 1 ml total volume of cocktail:
 - a. Add 1-20 μg DNA (sterile TE, pH 8.0) in 0.450 sterile water. (DNA was sterilized by ethanol precipitation.)
 - b. Add 0.500 ml of 2X HBS, mix well.
 - c. With manual pipette with autoclaved disposable tips, squirt 0.050 ml of 2.5 M CaCl_2 and vortex immediately.
 - d. Allow the mixture to left undisturbed for 15-30 minutes at room temperature. If precipitate has not formed use a sterile cotton-plugged 1 ml pipette to bubble into the mixture, the precipitate usually forms.

- e. 1 ml of transfection cocktail is added directly to recipient dish containing 10 ml media.
4. The dishes containing the DNA precipitate are incubated at 37°C for 12 hours.
5. Media with the precipitate is removed and fresh media is added.
6. Cells are prepared for irradiation 12 hours post-transfection, or selected for drug resistance 24 hours post-transfection.

APPENDIX I: CELL LINES

All the cell lines and bacterial strain are gifts from Michael Botchan.

I. HB101. This E. coli hybrid is a commonly used recipient in transformation and for the large scale growth and purification of plasmids. Its genotype is: $\text{Pro}^- \text{leu}^- \text{thi}^- \text{lacY}^- \text{hsdR}^- \text{endA}^- \text{recA}^-$, rpsL20 , ara-14 galK2xyl-5 mtl-1 supE44 (Boyer and Rousland-Dussolx 1969; Bolivar et al. 1977).

II. MAMMALIAN CELL LINES

A. CV-1. The CV-1 line was derived from the kidney of a male adult African green monkey by F. Jensen, et al. (1964) for use in Rous sarcoma virus transformation studies. This cell line is routinely used for the production of SV40 virus which produces a lytic infection.

B. COS-7. This cell line is derived from the transfection of a CV-1 cells with a SV40 DNA plasmid that contains a defective origin of replication (Gluzman, 1981). This cell line is SV40-A gene transformed, and produces T-antigen. This cell line supports the replication of SV40 mutants in the A-gene. Indeed, it supports the replication of the 381-bp Eco RII restriction fragment that contains the SV40 origin of replication.

C. Rat-2. This cell line is derived from an established rat fibroblastoid cell line F2408 (Prasad, et al., 1976) that has been selected for BuDr resistance (Topp, 1981). This cell

line is deficient in thymidine kinase activity, non-permissive for SV40 infection, flat, highly transfectable by exogenous DNA, and can tolerate 39°C incubations.

- D. Ltk-. This cell line is derived from LM cells that were selected for BuDr resistance (Kit, et al., 1963). This cell line is highly transfectable by exogenous DNA, and has never been shown to revert spontaneously or by the action of mutagens to the tk⁺ phenotype. This cell line is often used for the production of DNA carrier for thymidine kinase transfections.

X	Y	X	Y
2.000	1.254	4.000	0.400
2.400	2.164	3.420	2.127
2.440	2.222	3.450	2.222
3.420	2.314	3.500	2.267
3.520	2.413	3.540	2.477
3.550	2.551	3.520	2.515
3.600	2.629	3.620	2.750
3.640	2.826	3.660	2.915
3.620	2.932	3.700	3.196
3.700	3.345	3.740	3.534
3.760	3.711	3.720	4.026
3.200	4.307	3.220	4.701
3.240	4.254	3.260	4.312
3.220	2.702	3.900	1.423
3.220	0.746	3.940	0.503
3.250	0.476	3.920	0.451
4.000	0.417	4.000	0.422
4.050	0.352	4.100	0.323
4.150	0.222	4.200	0.272
4.250	0.235	4.300	0.220
4.250	0.157	4.400	0.120
4.450	0.159	4.500	0.154
4.550	0.140	4.600	0.132
4.650	0.125	4.700	0.119
4.750	0.111	4.200	0.104
4.250	0.099	4.900	0.093
4.250	0.090	5.000	0.085

FILENAME • LF:E225,206J549-330R.DAT
 AVISHAI BRAGG CURVE 23-APP-83 23:19:51 BIOLOGY BENCH
 APAR • 40.00 ZPAR • 19.00 BEVATRON EAMU • 330.00
 2/64 PB SCATTERER 0.00 2ND SCATTERER 0.00 CM SPRL PDGE FLTR
 SEM ON FRONT BENCH REMOVED

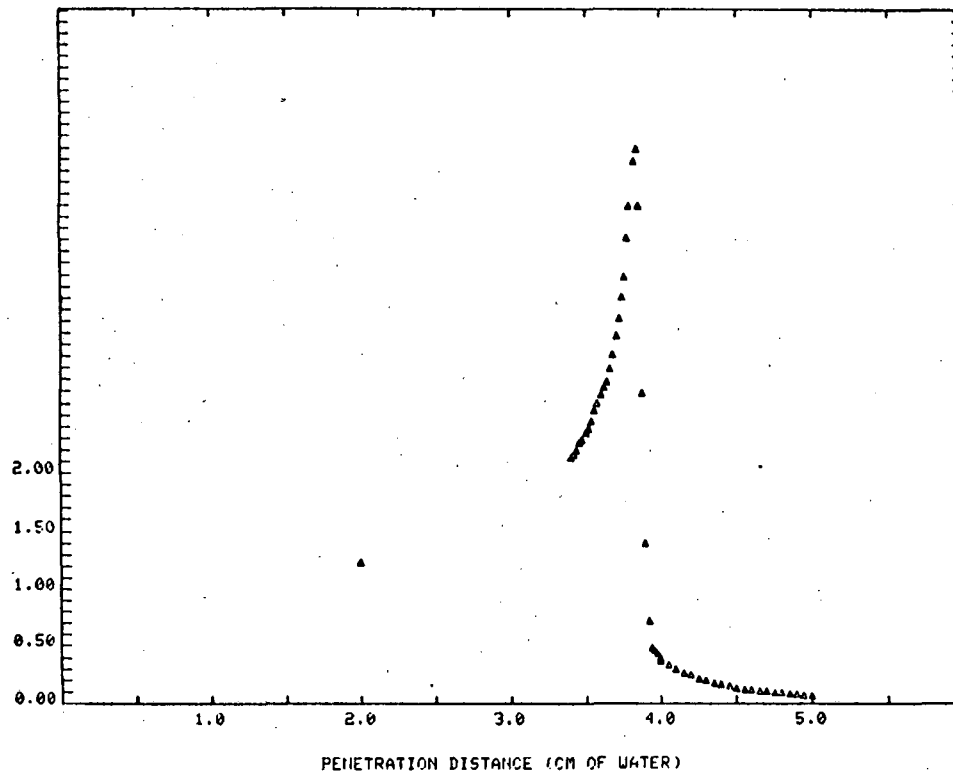


Figure A.7. Bragg curve of 330 Mev/amu argon ions.

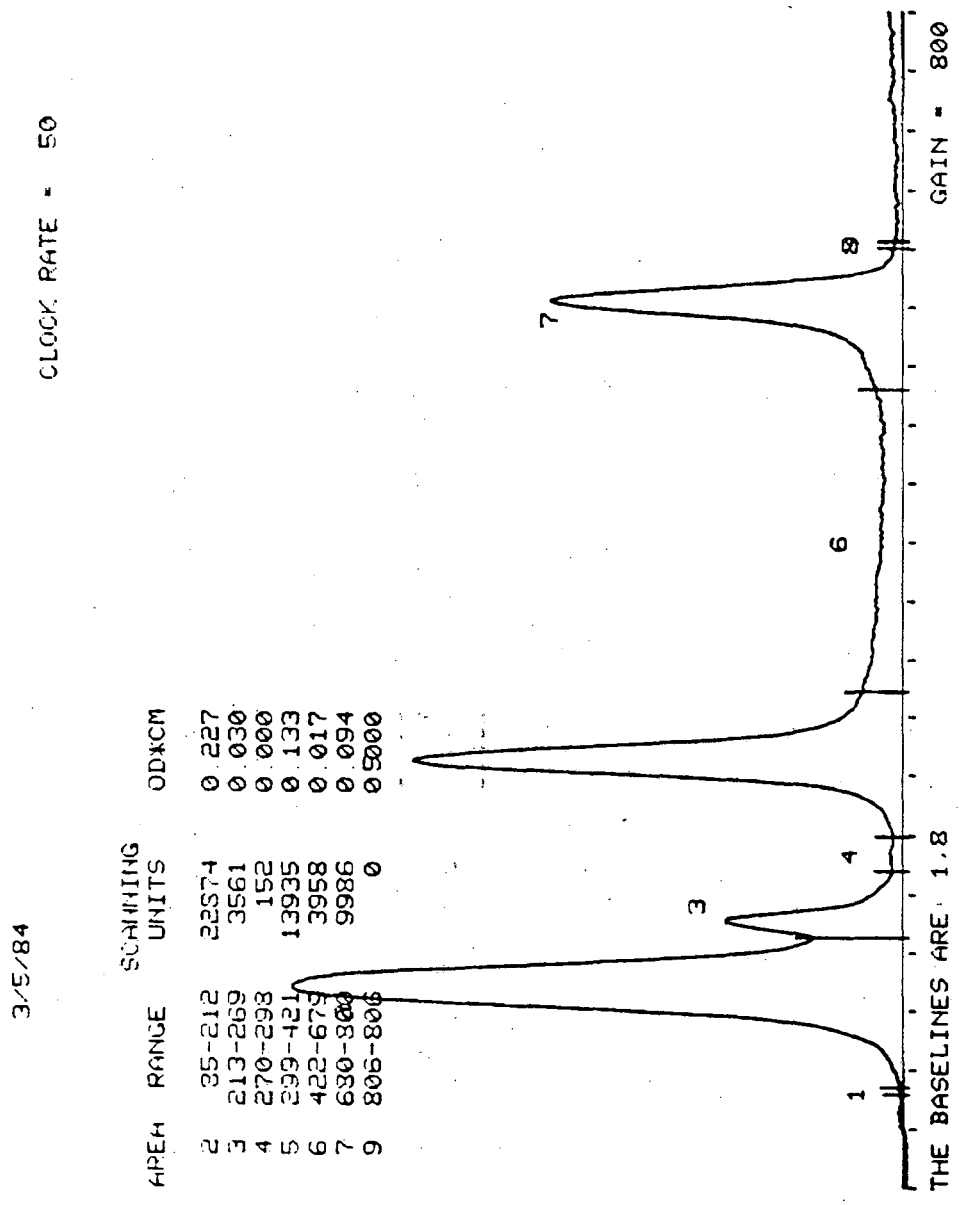


Figure A.8. Densitometer scan of Hirt extract from unirradiated cells.

3/5/84

CLOCK RATE = 50

AREA	RANGE	SCANNING UNITS	OD*CM
2	50-199	23767	0.237
3	200-258	6472	0.061
4	259-290	471	0.002
5	291-400	15549	0.151
6	405-752	13399	0.100

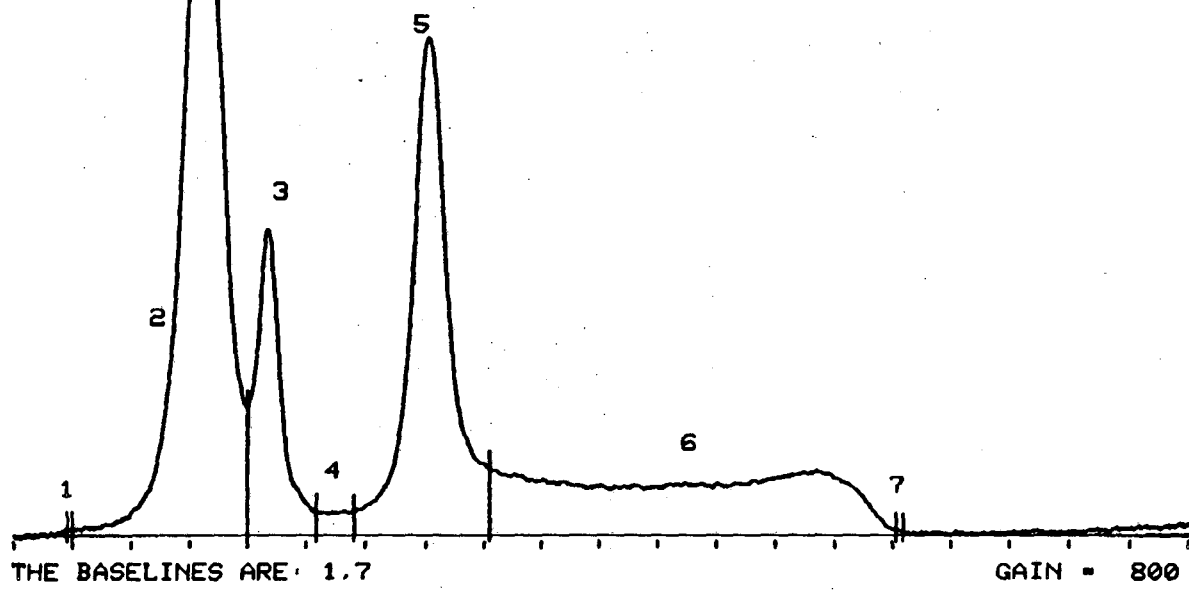


Figure A.9. Densitometer scan of Hirt extract from irradiated (5.00 Grays) cells.

3/5/84
CLOCK RATE • 50

AREA	RANGE	SCANNING UNITS	ODXCM
2	229-405	6330	0.043

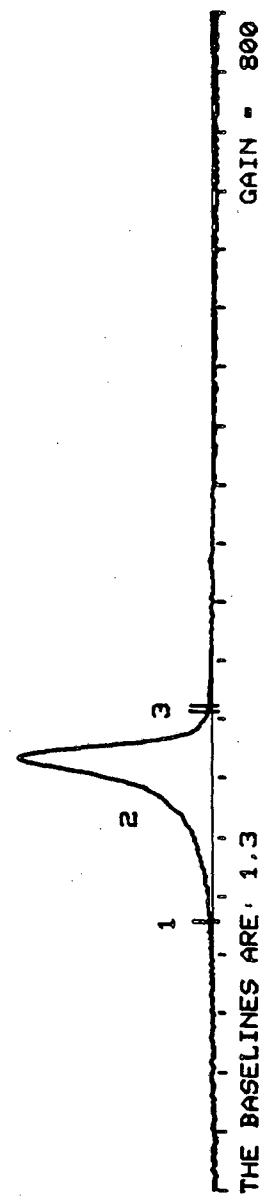


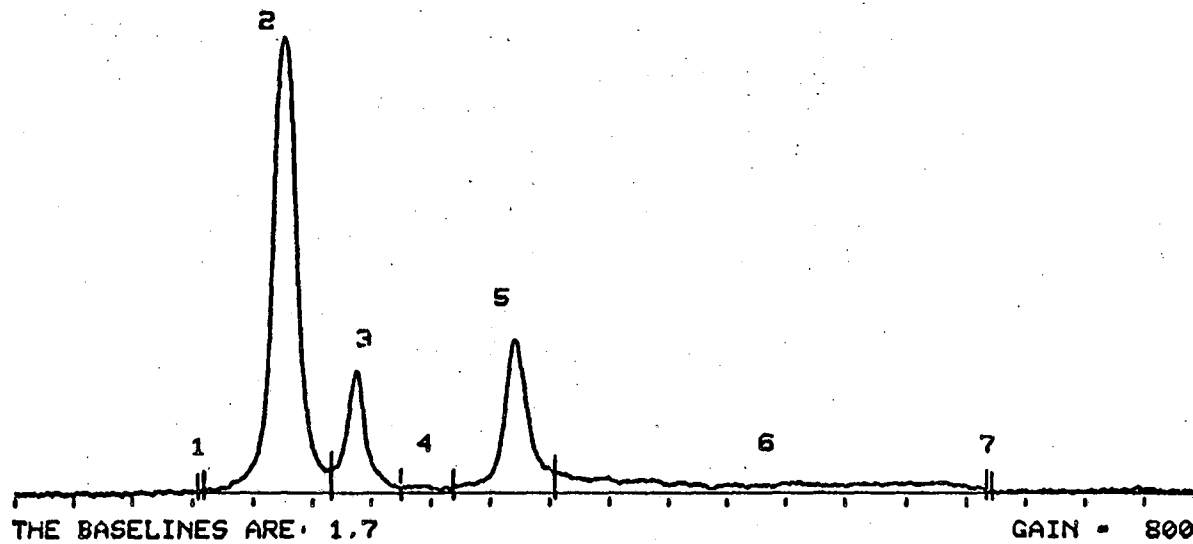
Figure A.10. Densitometer scan of 150 pg of linearized pTK2 DNA.

Figure A.11. Densitometer scan of Hirt extract from unirradiated nuclei.

3/5/84

CLOCK RATE = 50

AREA	RANGE	SCANNING UNITS	OD*CM
2	153-265	9617	0.087
3	266-324	2056	0.013
4	325-368	127	0.000
5	369-454	3193	0.022
6	455-816	2010	0.000



3/5/84

CLOCK RATE = 50

AREA	RANGE	SCANNING UNITS	OD*CM
2	156-261	10135	0.092
3	262-326	2473	0.017
4	327-362	178	0.000
5	363-459	3210	0.021
6	460-746	1261	0.000
8	748-749	2	0.000

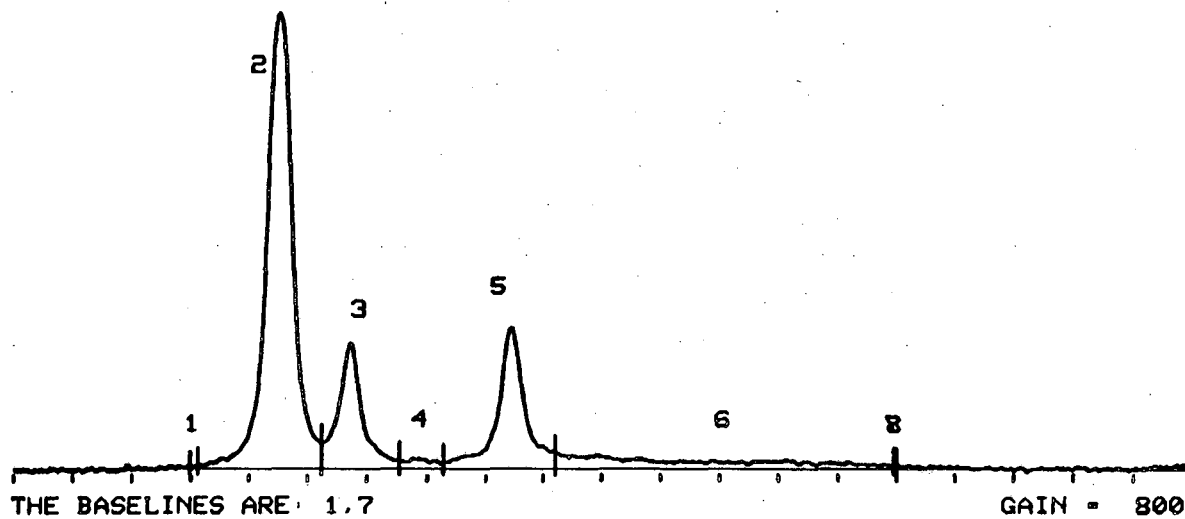


Figure A.12. Densitometer scan of Hirt extract from irradiated (5.00 Grays) nuclei.

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