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EDITED BY

G. H. BOURNE J. F. DANIELLI

ASSISTANT EDITOR
K. W. JEON

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Contents

List of Contributors vii

Integration of Oncogenic Viruses in Mammalian Cells

Carlo M. Croce

I. Introduction 1
II. Somatic Cell Hybridization Techniques to Map Human Genes 2
III. Somatic Cell Hybrids to Map Viral Integration Sites 2
IV. Expression of the Transformed Phenotype and of Tumorigenicity in Hybrids between Normal Rodent Cells and SV40 Transformed Human Cells 6
V. Integration of Other Oncogenic Viruses in Transformed Cells 6
VI. Does the Integration of Tumor Virus DNA in the Chromosomal DNA Occur by DNA Homology? 10
VII. DNA-Mediated Cell Transformation of Mouse Teratocarcinoma Cells 11
VIII. Conclusion 14
References 16

Mitochondrial Genetics of *Paramecium aurelia*

G. H. Beale and A. Tait

I. Introduction 19
II. The Mitochondrial Genome 21
III. Inheritance of Mitochondrial Characters 27
IV. Biochemical Products of Mitochondrial Genes 34
V. Conclusion 36
References 38

Histone Gene Expression: Hybrid Cells and Organisms Establish Complex Controls

Philip Hohmann

I. Introduction 41
II. The Histones 43
III. The Histone Genes 50
IV. General Features of the Control of Histone Gene Expression 56
V. The Nature of Gene Control in Hybrid Organisms and Cells 64
VI. Cell Replication and Histone Synthesis in Hybrids 75
References 88

Gene Expression and Cell Cycle Regulation

STEVEN J. HOCHHAUSER, JANET L. STEIN, AND GARY S. STEIN

I. Introduction . . . 96
II. Techniques and Approaches Used to Study the Cell Cycle . . . 104
III. Models for Cell Cycle Regulation . . . 120
IV. Ultrastructural Changes during the Cell Cycle . . . 130
V. Biochemical Changes in Quiescent Cells Stimulated to Proliferate . . . 136
VI. Biochemical Changes during the Cell Cycle of Continuously Cycling Cells . . . 162
VII. The Application of Cell Cycle to Biological Problems . . . 199
VIII. Summary and Prospects for Future Research . . . 209
References . . . 212

The Diptera as a Model System in Cell and Molecular Biology

ELENA C. ZEGARELLI-SCHMIDT AND REBA GOODMAN

I. Introduction . . . 246
II. Chromatin Structure . . . 247
III. Middle Repeated DNA (MR DNA) . . . 265
IV. Highly Repeated DNA (HR DNA), Satellites, and Heterochromatin . . . 289
V. Puffs . . . 299
VI. Heat Shock . . . 316
VII. Mechanisms of Gene Control: The Nonhistone Proteins . . . 329
References . . . 354

Comments on the Use of Laser Doppler Techniques in Cell Electrophoresis: Reply to Pretlow and Pretlow's Review

JOEL H. KAPLAN AND E. E. UZGIRIS

I. Introduction . . . 365
II. The Method . . . 365
III. Lymphocyte Studies . . . 368
References . . . 371

Comments on the Use of Laser Doppler Techniques as Represented by Kaplan and Uzgiris: Reply to Kaplan and Uzgiris

THOMAS G. PRETLOW II AND THERESA P. PRETLOW

Text . . . 373
References . . . 379

INDEX . . . 381

INTERNATIONAL REVIEW OF CYTOLOGY, VOL. 71

Integration of Oncogenic Viruses in Mammalian Cells

CARLO M. CROCE

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I. Introduction 1
II. Somatic Cell Hybridization Techniques to Map Human Genes . . 2
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V. Integration of Other Oncogenic Viruses in Transformed Cells . . 6
VI. Does the Integration of Tumor Virus DNA in the Chromosomal DNA Occur by DNA Homology? 10
VII. DNA-Mediated Cell Transformation of Mouse Teratocarcinoma Cells 11
VIII. Conclusion 14
References 16

I. Introduction

Cells transformed by either RNA- or DNA-containing oncogenic viruses retain the genomes of the transforming viruses and, in general, express antigens, coded, at least in part, by the viral genomes (Black *et al.*, 1963; Tegtmeyer, 1974; Lai and Nathans, 1974; Cooper and Temin, 1974). Nucleic acid hybridization techniques have been used to determine the state of the genomes of the tumor viruses such as Simian virus 40 (SV40) in transformed cells (Sambrook *et al.*, 1968). These studies indicated that viral DNA sequences are integrated in the cellular DNA of viral-transformed cells (Sambrook *et al.*, 1968; Croce *et al.*, 1973, 1974). Since these early studies additional investigations have shown that all cells transformed by oncogenic DNA tumor viruses and by RNA tumor viruses such as avian and murine sarcoma and leukemia viruses contain viral DNA sequences integrated in the cellular DNA (Sambrook *et al.*, 1968; Varmus *et al.*, 1973; Kettner and Kelly, 1975; Botchan *et al.*, 1976). In the case of RNA-containing viruses, DNA copies of the viral RNA are transcribed by viral RNA-dependent DNA polymerases (Temin, 1971; Temin and Baltimore, 1972) and the resulting complementary DNAs become covalently integrated in the cellular DNA (Varmus *et al.*, 1973, 1974). Deoxyribonucleic acid, derived from mammalian cells transformed by the RNA-containing Rous Sarcoma virus, has also

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been shown to contain the genome of the virus since it could be used to transfer the viral genetic information into uninfected chicken cells (this virus is of avian origin and can replicate only in certain chicken cells) (Hill and Hillova, 1972; Hill *et al.*, 1974; Cooper and Temin, 1974). Genomes of RNA-containing tumor viruses are present, however, not only in transformed cells, but also in normal cells (Huebner *et al.*, 1970; Aaronson *et al.*, 1969; Lowry *et al.*, 1971; Hanafusa *et al.*, 1974). These genomes appear to segregate in mammalian fashion (Rowe, 1972, 1973; Rowe and Hartley, 1972; Rowe and Pincus, 1972; Rowe *et al.*, 1972a,b) and, therefore, appear to be part of the genetic make-up of most of mammalian cells with the possible exception of those of human origin, since *no bona fide* human RNA tumor viruses have been discovered at the present time.

We intended to investigate the state of the viral genome in virus transformed cells in order to determine:

1. Whether the viral genomes are chromosomally integrated in transformed cells.
2. Whether the integration of the viral genome is chromosome specific.

For this purpose we used somatic cell hybridization techniques.

II. Somatic Cell Hybridization Techniques to Map Human Genes

Somatic cell hybrids between human and rodent cells have been used to map human genes to their specific chromosomes (Ruddle, 1973; Croce *et al.*, 1979). This was made possible by the finding that rodent–human hybrids, in general, segregate human chromosomes (Weiss and Green, 1967; Croce, 1976). If the expression of a human specific phenotype such as an enzyme segregates concordantly with a specific human chromosome in a relatively large number of independent rodent–human hybrids, it can be concluded that the gene coding for that phenotype is located on that human chromosome. In Table I we report the results of the analysis of a series of mouse–human hybrids for the expression of the human form of the enzyme β-glucuronidase (Chern and Croce, 1975). As shown in Table I, the expression of the human enzyme segregated concordantly with the retention of human chromosome 7. Therefore, the gene coding for β-glucuronidase can be mapped on this human chromosome.

III. Somatic Cell Hybrids to Map Viral Integration Sites

Sambrook *et al.* (1968) have determined that the DNA-containing tumor virus SV40 is integrated into the cellular DNA of transformed cells. Since human

TABLE I
CONCORDANT SEGREGATION OF HUMAN β-GLUCURONIDASE EXPRESSION WITH THE PRESENCE OF CHROMOSOME 7

	Human chromosome 7	
	+	−
Human β-glucuronidase		
+	15	0
−	0	12

cells can be transformed by SV40 (Girardi *et al.*, 1965) we produced somatic cell hybrids between rodent cells and SV40 transformed human cells and studied the hybrids for the expression of SV40 T antigen, which is coded by the A gene of SV40 (Black *et al.*, 1963) and for the presence of human chromosomes. As shown in Table II the first two SV40 transformed human cell lines we have studied contained SV40 integrated in the human chromosome 7. The expression of the T antigen segregated with this human chromosome and with no other human chromosome in a large number of independent hybrid clones (Table II) (Croce *et al.*, 1973). In order to confirm this finding, we subcloned three SV40 T antigen positive hybrid clones, one of which (52-61(1) Cl 5 BUdR) contained only human chromosome 7 and no other human chromosome (Table III) (Croce and Koprowski, 1974a). We also rehybridized cells of this hybrid line that were deficient in thymidine kinase with mouse cells deficient in hypoxanthine phosphoribosyltransferase. The subclones and the triple hybrids segregated into T antigen positive and negative cells. The expression of SV40 T antigen segregated concordantly with the presence of human chromosome 7 in the hybrid subclones and triple hybrids (Table IV) (Croce and Koprowski, 1974b). We have also produced mouse × human somatic cell hybrids with the SV40 transformed cell line GM54VA. In these hybrids the expression of SV40 T antigen segregated

TABLE II
[illegible]NT SEGREGATION BETWEEN HUMAN CHROMOSOME C-7 AND SV40 T A[illegible]

	All hybrids	
	+ C-7	−
SV40 T antigen		
+	71	0
−	3[a]	12

[a] Exceptions are three LN-SV × C1-1D hybrid clones that contain the C-7 chromosome, but are negative for T antigen.

TABLE III

EXPRESSION OF SV40 T ANTIGEN IN MOUSE-HUMAN HYBRID SUBCLONES AND TRIPLE HYBRIDS

Hybrid clones	Number of subclones or triple hybrids	Number of T antigen positive subclones or triple hybrids
52-58 Cl 19	15	3
52-62 (1) Cl 5 BUdR	9	7
52-62 (1) Cl 16 BUdR	5	4
52-62 (1) Cl 5 BUdR × IR	20	12

concordantly with the presence of human chromosome 17 (Table V) (Croce, 1977). In the line GM637 that contained two SV40 integration sites, we have assigned one of the two integration sites to human chromosome 12 (M. Shander, E. DeJesus, and C. M. Croce, in preparation). This was made possible by the finding that a *Sac*I 19 kb fragment containing SV40 DNA segregated concordantly with human chromosome 12 in mouse × human hybrids. The second integration site is not in human chromosome 12 and is not in human chromosome 8. Recently, Kucherlapati *et al.* (1978) have assigned the SV40 integration site to human chromosome 8 in this cell line. From these studies it can be concluded that the gene coding for SV40 T antigen is located in the chromosomal DNA of virus-transformed cells and that this gene can be located on different chromosomes in different SV40 transformed cells. Since we were able to recover infectious SV40 only from SV40 T antigen positive mouse hybrid cells following fusion with permissive African green monkey cells we concluded that the chromosome that coded for SV40 T antigen contained the entire genome of the virus (Croce *et al.* 1974). These results were confirmed by experiments of

TABLE IV

POSITIVE CORRELATION BETWEEN THE PRESENCE OF SV40 T ANTIGEN AND HUMAN CHROMOSOME 7 IN 29 SUBCLONES OF THREE T ANTIGEN POSITIVE MOUSE-HUMAN HYBRID CLONES AND IN 20 TRIPLE HYBRID CLONES DERIVED FROM THE FUSION OF 52-62 (1) Cl 5 BUdR WITH MOUSE IR CELLS

All hybrid subclones and triple hybrids		
	Human chromosome 7	
	+	−
SV40 T antigen		
+	26	0
−	0	23

TABLE V

EXPRESSION OF SV40 T ANTIGEN IN HYBRIDS BETWEEN A3 TK-CHINESE HAMSTER CELLS AND GM54VA SV40 TRANSFORMED HUMAN CELLS

Hybrid clones	Selected in HAT		Counterselected in BUdR	
	Percentage T antigen positive cells	Number of hybrid cells containing human chromosome 17/total analyzed	Percentage T antigen positive cells	Number of hybrid cells containing human chromosome 17/total analyzed
56-11 Cl 1	>90	15/15	ND[a]	ND
56-11 Cl 2	80	10/11	<1	0/10
56-11 Cl 5	>90	12/12	<1	0/12
56-11 Cl 8	75	9/10	ND	ND
56-11 Cl 9	50	13/13	<1	0/10
56-11 Cl 11	<1	15/15	<1	0/10
56-11 Cl 14	>80	13/14	ND	ND
56-11 Cl 15	>90	15/15	<1	0/10
56-11 Cl 16	<1	13/14	ND	ND
56-11 Cl 17	>90	12/12	<1	0/15
56-11 Cl 18	>90	12/13	<1	0/10
56-11 Cl 24	>90	12/12	<1	0/11
56-11 Cl 29	>90	11/11	<1	0/10
56-11 Cl 30	>90	11/13	ND	ND
56-11 Cl 31	<1	12/12	ND	ND

[a] Not done.

DNA–DNA reassociation kinetics using labeled viral DNA as a probe (Khoury and Croce, 1975). The number of copies of SV40 DNA corresponded to the number of copies of the human chromosome carrying the SV40 genome to the hybrid cells (Khoury and Croce, 1975). In this case, the parental human cell line was LN-SV in which the SV40 genome is integrated in human chromosome 7 (Khoury and Croce, 1975; Campo *et al.*, 1978).

Recently the Southern transfer method (1975) has been applied to the study of the integration of SV40 in different transformed cells (Kettner and Kelly, 1976). Results of these studies indicated that the viral DNA is inserted in different sites in different SV40 transformed rodent cells (Kettner and Kelly, 1976; Botchan *et al.*, 1976). Study of mouse–human hybrids with LN-SV human cells by the same method confirmed that SV40 is integrated in human chromosome 7 in this cell line (Campo *et al.*, 1978).

IV. Expression of the Transformed Phenotype and of Tumorigenicity in Hybrids between Normal Rodent Cells and SV40 Transformed Human Cells

In order to determine whether the integration of SV40 in a human chromosome results in the expression of the transformed phenotype and in tumorigenicity, SV40 transformed human cells containing the SV40 genome integrated in either human chromosome 7 or 17 were hybridized with cells directly derived from the mouse such as peritoneal macrophages. Interestingly, all the hybrids with the human cell line (LN-SV) carrying the SV40 genome in human chromosome 7 (Figs. 1 and 2) retained human chromosome 7 and all the hybrids with the human cell line carrying the SV40 genome in human chromosome 17 G54VA retained human chromosome 17 (Fig. 3) (Croce and Koprowski, 1974b, 1975; Croce, 1977) whereas all the other human chromosomes segregated in the hybrid clones. All hybrids were expressing SV40 T antigen, were transformed *in vitro*, and were capable of forming tumors in immunosuppressed animals such as nude mice (Croce *et al.* 1975a,b; Koprowski and Croce, 1977). The presence of a single copy of the chromosome carrying the SV40 genome was sufficient for the expression of the transformed phenotype and for the tumorigenicity of the hybrids (Figs. 4 and 5) (Croce *et al.*, 1975b). These results indicate that the chromosomes containing the viral genomes are cancer chromosomes that make the cells transformed and tumorigenic.

V. Integration of Other Oncogenic Viruses in Transformed Cells

Evidence that polyoma virus, another small DNA tumor virus (Soeda *et al.*, 1979; Novak *et al.*, 1980), certain adenoviruses (Doerfler *et al.*, 1974), and

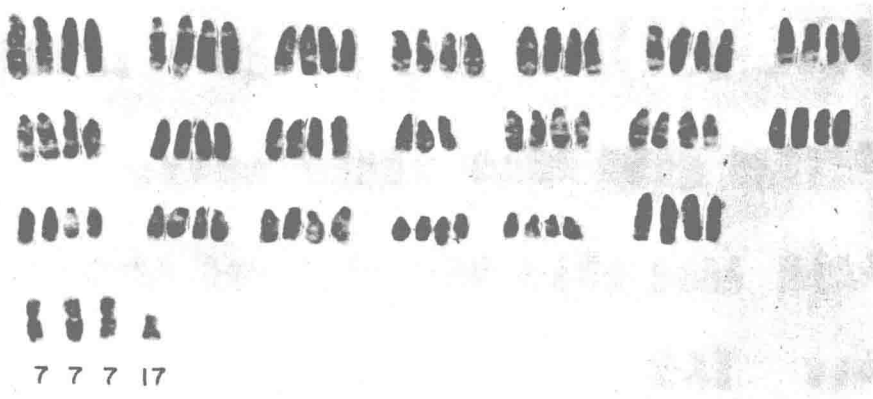

FIG. 1. Karyotype of a hybrid cell between a mouse peritoneal macrophage (MPM) and an LN-SV SV40 transformed human cell. This hybrid has retained three copies of human chromosome 7 and one copy of human chromosome 17.

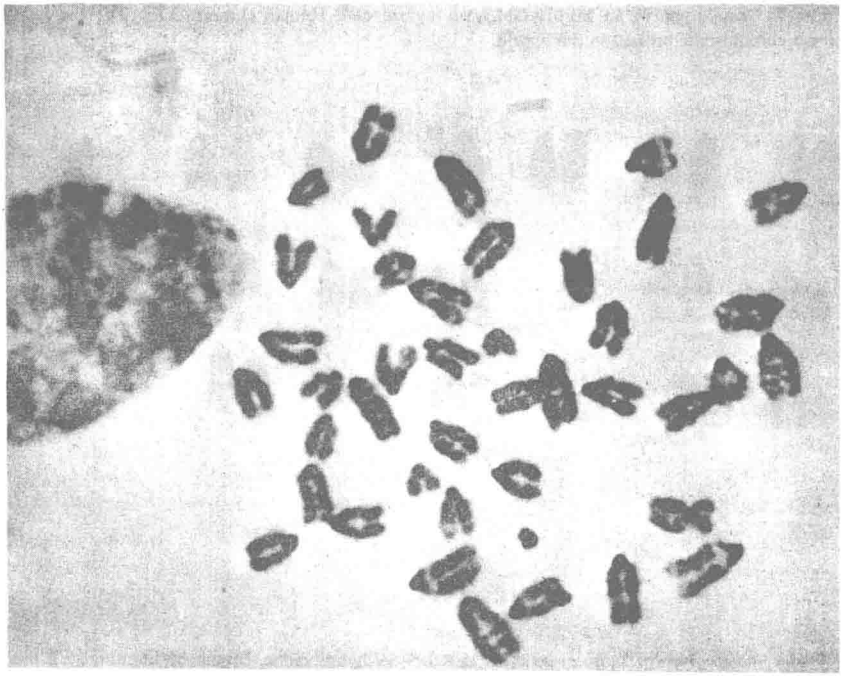

FIG. 2. Metaphase plate of an MPM × LN-SV hybrid that contains only chromosome 7 and no other human chromosome.

FIG. 3. Karyotype of an MPM×GM54VA hybrid cell. Human chromosome 17 is the only human chromosome present in the hybrid.

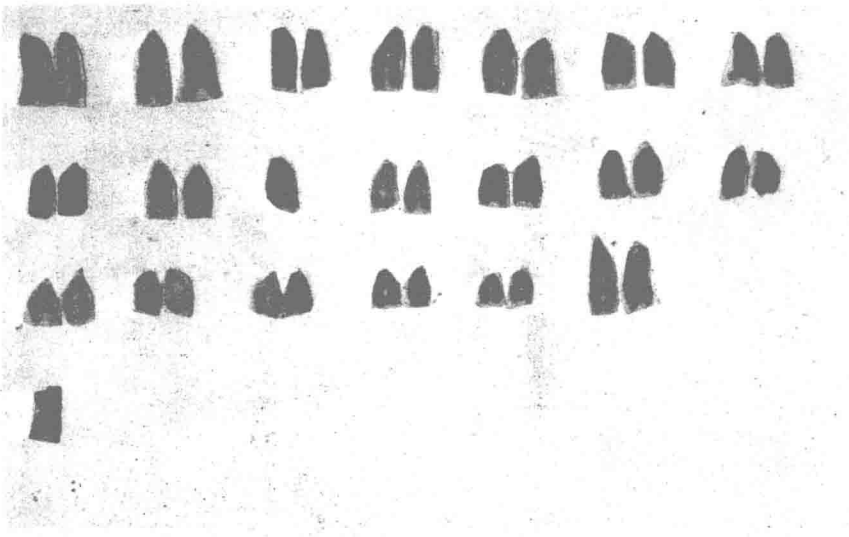

FIG. 4. Karyotype of a tumorigenic MPM × LN-SV hybrid clone. Human chromosome 7 is the only human chromosome present in the hybrid.

FIG. 5. Tumor formed by the injection of an MPM × LN-SV hybrid into a nude mouse.

RNA tumor viruses (Varmus *et al.* 1973, 1974) are also integrated in the cellular genome of viral transformed cells has been obtained. It is, however, not known where the chromosomal location of these viral genomes is or whether the integration occurs at specific sites or at random. The fact that the viral genomes are located in different restriction enzyme fragments in virus transformed cells does not necessarily mean that the integration occurs at random. It is possible, in fact, that homologous DNA sequences that are repeated on different chromosomes and/or on the same chromosome are the sites of integration.

Conclusive information on this subject will be derived by studies of the nucleotide sequences of cloned viral integration sites.

In the case of Epstein–Barr (EB) virus, a herpes virus that has been considered as the causative agent of Burkitt lymphoma, a neoplastic condition observed in certain areas of Africa (Epstein *et al.*, 1964), disagreement exists on whether the viral DNA or segments of it are integrated in the cellular DNA of the malignant cells. Klein and his associates reported some experiments that suggested that the genome of the virus is not covalently integrated in the cellular DNA but it is associated with the chromosomal DNA by alkaline labile bonds in Burkitt lymphoma cells (Adams *et al.*, 1973). Other investigators have reported that the expression of the EB viral induced nuclear antigen (EBNA) (Reedman and Klein, 1973), is associated with chromosome 14 in hybrids between mouse cells and Burkitt lymphoma cells (Yamamato *et al.*, 1978). We have also attempted to

locate the genome of EB virus in Burkitt lymphoma cells using hybrids between mouse fibroblasts and human Burkitt lymphoma cells (Glaser *et al.*, 1978). The results of this analysis indicated that the expression of EBNA and the presence of viral DNA sequences are not associated with any human chromosomes in these hybrids (Glaser *et al.*, 1978). These results suggest that some of the viral DNA is an episomal state in Burkitt lymphoma cells. It is possible, however, that EB viral DNA sequences are also integrated in the chromosomal DNA of these malignant cells. It will become feasible to resolve this question when the DNA fragments of the entire EBV genome will be cloned in plasmid or phage cloning vectors. The use of these cloned DNA probes to analyze the DNA of Burkitt lymphoma cells containing a very limited number of EB viral genome equivalents as part of it should answer this question.

VI. Does the Integration of Tumor Virus DNA in the Chromosomal DNA Occur by DNA Homology?

We have started to address the question of whether the integration of SV40 and polyoma virus DNA in the chromosomal DNA occurs by DNA homology. We chose one somatic cell hybrid clone between thymidine kinase-deficient mouse cells (LM-TK) and GM637 SV40 transformed human cells that contain two SV40 genomes, one of which is integrated in human chromosome 12. Southern blotting analysis of the hybrid cellular DNA following restriction with the enzyme *Sac*I indicated that the human parental cells and some of the hybrids contained SV40 in a 19 kb DNA fragment (M. Shander and C. M. Croce, unpublished results). T antigen positive hybrid cells that contained the *Sac*I 19 kb DNA fragment hybridizing with SV40 DNA were counterselected in medium containing high concentrations of 5-bromodeoxyuridine and clones that were T antigen positive and thymidine kinase-deficient were obtained. Such T antigen positive thymidine kinase deficient mouse-human hybrids were then transformed with recombinant plasmids containing the gene for herpes simplex type 1 thymidine kinase and the entire SV40 genome (Fig. 6) (Linnenbach *et al.*, 1980) and transformants were selected in HAT selective medium (Littlefield, 1964) in which only thymidine kinase positive cells can grow. Transformants were obtained and we are presently determining whether the exogenous recombinant SV40 molecules integrate where the endogenous SV40 DNA is located (on chromosome 12). We will restrict the transformant cellular DNA with *Sac*I and other restriction enzymes and we will establish whether the *Sac*I 19 kb restriction fragment disappears in the transformants.

The use of a thymidine kinase vector could be applied to the study of integration of other oncogenic viruses in order to establish conclusively whether integration of the viral genome in the cellular genome occurs by DNA homology.

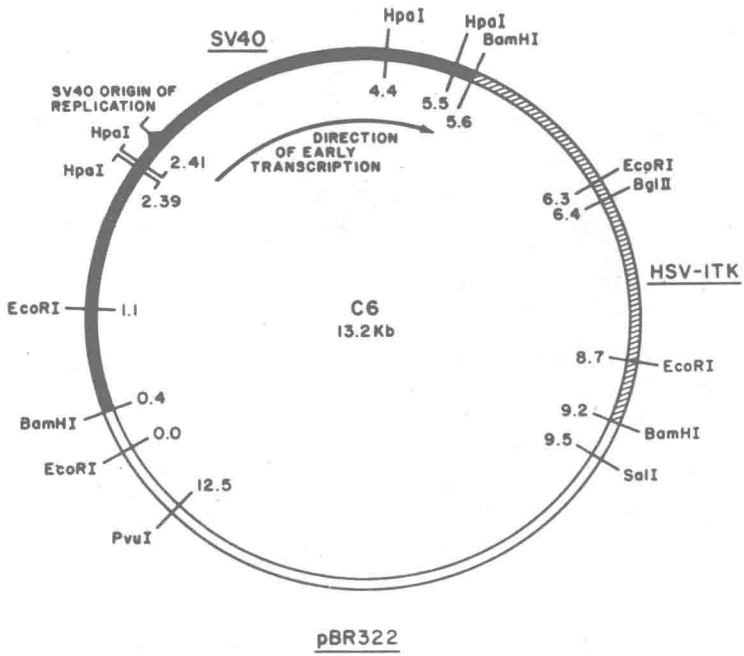

FIG. 6. Restriction enzyme map of the plasmid C6 containing the entire genome of SV40 and the 3.4 kb fragment of herpes simplex virus type 1 carrying the gene for thymidine kinase into the plasmid pBR322.

VII. DNA-Mediated Cell Transformation of Mouse Teratocarcinoma Cells

Mouse teratocarcinoma stem cells do not express SV40 or polyoma virus T antigen following viral infection (Swartzengruber and Lehman, 1975). On the contrary, infection of differentiated cells with these viruses results in the expression of the viral (tumor) T antigens (Swartzengruber *et al.*, 1977; Seegal and Khoury, 1979; Seegal *et al.*, 1979). Apparently, the block does not occur at the level of penetration and viral uncoating in the cellular nuclei (Swartzengruber *et al.*, 1977; Seegal and Khoury, 1979; Seegal *et al.*, 1979) but it involves either the transcription of the viral genes or the processing of viral-specific mRNAs. In order to determine the molecular basis of the lack of expression of SV40 T antigen in mouse teratocarcinoma cells we transformed TK-F9 mouse teratocarcinoma cells with a C6 plasmid vector carrying the gene for herpes simplex virus thymidine kinase and the entire SV40 genome (Fig. 6) (Linnenbach *et al.*, 1980) in the plasmid pBR322 by the DNA-mediated gene transfer approach. As shown in Fig. 7 the transformants selected in HAT medium expressed herpes simplex virus thymidine kinase.