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TUMOR VIRUSES AND EARLY MOUSE EMBRYOS

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Abbreviations: SV40, simian virus 40; MuLV, murine leukemia virus; MSV, murine sarcoma virus; Mu-MTV, mouse mammary tumor virus; MVM, minute virus of mice; VSV, vesicular stomatitis virus; MCMV, mouse cytomegalovirus; EMV encephalomyocarditis virus; prefixes to these viruses refer to the strains originally isolated by Moloney (Mo), Rauscher (Ra), Friend (F), Abelson (A), Harvey (Ha) and Kirsten (Ki); IAP, intracisternal type A particles; PYS, parietal yolk sac; TK, thymidine kinase; TSTA, tumor-specific transplantation antigen.

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1. Introduction

IA. General

Studies of host-virus relationships during embryogenesis have been stimulated by the hope that they might contribute to the elucidation of the mechanisms by which embryonic cells enter into progressively more differentiated states. The potential usefulness of viruses for the study of differentiation processes was recognized clearly in the early sixties [1]. In a pioneering series of experiments, Saxen and coworkers [2,3] (see also Refs. 4–8) showed that the susceptibility of embryonic cells to viruses depended on their developmental state. While these experiments further substantiated previous indications that the teratogenic effects of any virus depend on its time of action during gestation they provided clear evidence that viral susceptibility could be used as a differentiation marker at the same time.

In the past 10 years, attention has been focussed on DNA and RNA oncogenic viruses and a detailed knowledge of their molecular structure has been obtained. Studies on the mechanisms by which oncogenic viruses integrate into the host genome as well as studies on interactions between host cell and virus products have led to important clues to the understanding of the mechanism of viral transformation. It also has become clear that oncogenic viruses constitute very useful tools in studies on normal cellular functions. They provide at first a model system to study the regulation of eucaryotic gene expression at a molecular level. More recently, they have allowed the definition of a number of cellular proteins which, in addition to their presumed role in transformation, might take part in normal physiological processes. Due to the progress in DNA recombinant techniques, the modifications of the viral genomes and the insertion into them of various types of foreign coding sequences is now at hand. While keeping their property of integrating into the cell genome such modified viruses may be powerful vectors for introducing genes into cells. At the same time, substantial progress has been made on the in vitro manipulation of mouse embryos, and a clearer picture of the relationships between the early cell lineages has emerged [10,241]. Furthermore, the development of studies with embryonal carcinoma cells has provided a very useful ersatz of normal early embryonic cells.

A new situation thus is created where the advances made in the field of oncogenic viruses may possibly be exploited in studies on development and where the questions delineated in the earlier studies on interactions between viruses and developing embryos may be tackled under more favorable conditions.

In this review we will report on experiments which deal with infection of early mouse embryos with DNA and RNA tumor viruses as well as with expression of various types of endogenous RNA viruses. An account of studies carried out on embryonal carcinoma cells will also be given.

IB. Background on tumor viruses

Before reviewing the main aspects of recent studies in this field, an account of the major properties of polyoma virus, SV40 and RNA tumor viruses will be given. Information concerning other viruses can be found in Fenner et al. [11]. A brief account of the main events of early mouse embryogenesis will also be provided. This should allow the reader not familiar with the virological or embryological fields to follow the experiments reported in this review.

IB-1. DNA tumor viruses: Simian virus 40 and polyoma virus

Simian virus 40 (SV40) and polyoma virus belong

to the papovavirus group. They are small DNA viruses, with a viral genome of about $3.5 \cdot 10^6$ daltons, constituted by a double-stranded, closed circular DNA molecule, divided equally into an early and a late region (Fig. 1). They lytically infect cells from their natural hosts, rhesus monkeys for SV40 and mice for polyoma virus. Mouse fibroblasts in culture thus will allow replication of polyoma virus. Mouse cells, however, are not permissive for SV40 and, upon infection with this virus, will express only early viral functions. Both viruses are oncogenic in certain rodents and induce malignant transformation of cultured cells from different mammalian species. The transformed cells contain viral sequences integrated into their own sequences, and express certain viral proteins, namely the early proteins also produced in the early phase of the lytic cycle. Expression of early (T) and late (V) proteins can be detected easily in the nuclei of single cells by indirect immunofluorescence tests using specific antisera. The complete nucleotide sequence of both viruses has been determined, and most, if not all, of the viral polypeptides have been identified. Considerable sequence homology, as well as similarities in the genetic organization of the two viruses, have been recognized, but there are also significant differences, in particular as regards the number of early proteins coded by the viruses and their presumed respective roles in transformation. The early region of SV40 codes for two proteins, large T and small t antigens, whereas polyoma virus codes for three proteins, large T, middle T and small t antigens. For further discussion of the role of these proteins in transformation, see the chapter 'Transformation by SV40 and polyoma virus' in Ref. 12. Differences also exist in a non-coding region present in both viruses around the origin of replication. It contains several sequences capable of forming secondary structures and, although its biological function is not clear at the present time, it is presumed to contain regulatory signals of importance for the

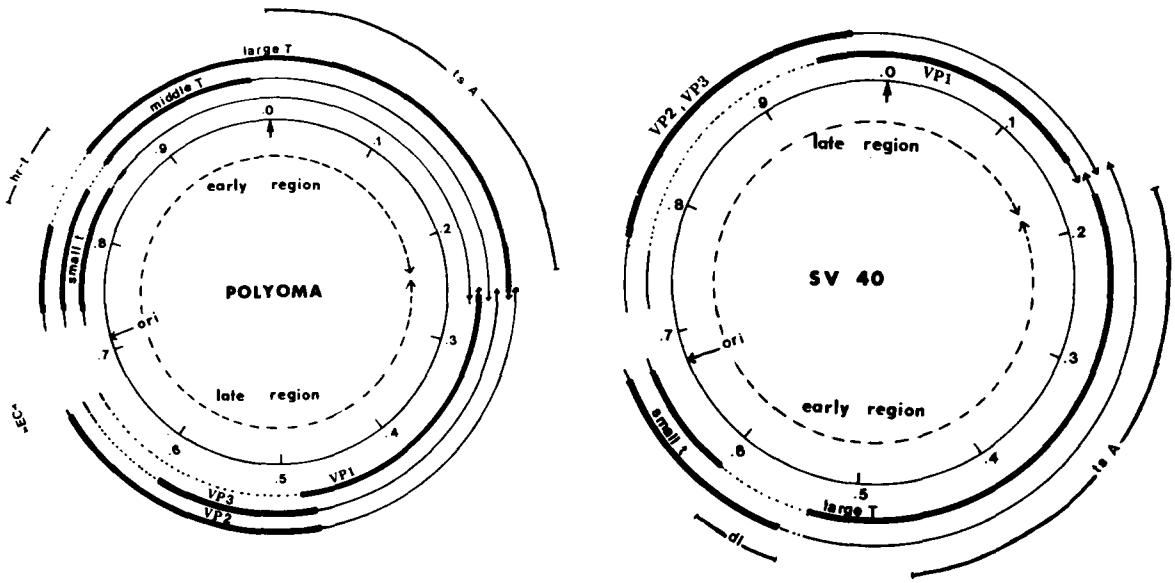

Fig. 1. Circular maps of the SV40 and polyoma virus genomes. The maps are oriented with the single cleavage site for the *Eco*RI restriction enzyme at 0.0. The two coding regions indicated by arrows in dotted lines are defined by the gene functions expressed primarily before (early) and after (late) viral DNA replication. Major mRNAs are represented by arrows originating from a region near the origin of viral DNA replication. The intervening sequences that are spliced out are indicated by dots. The mRNAs for the VP2 and VP3 late products of SV40 have identical sizes and have been represented by a single line. Protein coding regions are depicted by thick lines on each mRNA. The regions of the genome in which mutations mentioned in this review have been mapped are also indicated: (1) mutations which affect transformation, dl 054-0.39 (dlF) and *tsA* for SV40, *hr-t* and *tsA* for polyoma virus. (2) mutations of polyoma virus which allow growth on embryonal carcinoma cells (ECPy) (see Subsection IVA).

initiation of viral transcription and possibly for post-transcriptional processes. It may also bear recognition sites for cellular proteins. In the case of SV40, a new protein, called the agnoprotein, the existence of which had been predicted on the basis of an open reading frame in this region, has been detected recently late in the lytic cycle [12]. Detailed information on these viruses is available in Tooze [13].

IB-2. Murine RNA tumor viruses

The murine RNA tumor viruses or retroviruses constitute a much more complex and heterogeneous group of viruses. On the basis of the fine structure of the virus particles, three categories have been distinguished: type A, B and C viruses [14].

Genetic information for all three classes of RNA viruses is contained in the cell genome of all strains of mice tested so far, as well as in most, if not all, vertebrates, and therefore is transmitted vertically through the germ line. The endogenous type B and C viruses can give rise to infectious viral progeny, which can then be transmitted horizontally to animals and cells in culture. Endogenous type A sequences can also be expressed as viral particles in a variety of cells, but all attempts to demonstrate infectious properties of the particles have failed.

IB-2a. Type C RNA viruses. In the mouse, type C RNA viruses known so far belong to the murine leukemia virus group. Several review articles dealing with various aspects of the genetics and molecular biology of retroviruses have been published recently [15–18], and the reader is referred to these reviews for additional information.

Since the first isolation of a murine leukemia virus (MuLV) from the AKR mouse strain was made by Gross in 1951, a number of other MuLVs, also capable of inducing leukemia upon inoculation into mice, have been obtained from different strains of mice with spontaneous or radiation-induced leukemias and from MuLV-induced tumors. The widely used strains of MuLVs isolated by Moloney (Mo-MuLV), Rauscher (Ra-MuLV) and Friend (F-MuLV) were obtained in this last way. Replication-defective viruses, such as the Moloney murine sarcoma virus (Mo-MSV) and the Abelson virus (A-MuLV) (which induce sarcomas and lymphomas, respectively), were also derived in a similar manner and have been shown to result from the substitution of mouse cellular sequences for part of the viral sequences. Similarly, the Harvey (Ha-MSV) and Kirsten (Ki-MSV) defective murine sarcoma viruses isolated from MuLV-induced tumors in rat contain rat-derived genetic sequences (Fig. 2).

The genome of MuLVs is composed of two identical subunits of single-stranded RNA of molecular weight $3 \cdot 10^6$ (i.e., 10000 nucleotides). It codes for internal structure proteins (the group-specific-associated antigens or *gag* proteins), an RNA-dependent DNA polymerase or reverse transcriptase and the major envelope glycoprotein, gp70 (Fig. 2). Upon infection of susceptible cells by MuLVs, reverse transcription of the viral RNA leads to the formation of linear and circular double-stranded DNA intermediates. Proviral DNA (presumably a closed circular DNA) integrates into host cell chromosomes and RNA is transcribed from the integrated proviral DNA.

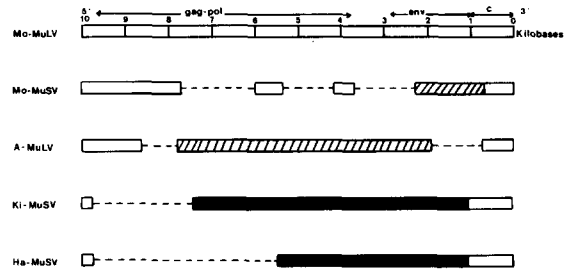

Fig. 2. Physical maps of genomic RNAs of Mo-MuLV and its replication defective derivatives, Mo-MuSV, A-MuLV, Ki-MuSV and Ha-MuSV. At the upper part of the figure, the linear map of the Mo-MuLV genome is represented. The structural genes are indicated. The *gag* gene codes for a precursor protein which is processed into the major internal structural proteins (including p30), the *pol* gene encodes the reverse transcriptase molecule and the *env* gene encodes for the major virion envelope glycoprotein gp70. The C region (common to all type C viruses) is presumed to play an important role in events related to proviral DNA synthesis, integration of the proviral DNA and regulation of transcription. The lower part of the figure represents the maps of the replication-defective viruses as indicated. The open blocks in the replication-defective genomes represent the corresponding segments from the Mo-MuLV genome. The hatched and closed blocks represent sequences derived from the genome of the host from which these viruses were first isolated, mouse and rat, respectively (adapted from Shih and Scolnick [18]). Additional data on A-MuLV are from Shields et al. [19].

Replication-defective MSVs will only replicate in the presence of an MuLV helper. The ends of the viral genome play a key role in virus replication and integration. It has been shown recently that the structure of the integrated provirus resembles that displayed by transposable elements from both procaryotes and eucaryotes ([20], and for a review, see Ref. 21).

All inbred laboratory mouse strains, as well as wild mice, tested so far have been shown to harbor multiple copies of type C viral genomes (from about 8 to 15 per haploid genome). These endogenous viral genes can be expressed to various degrees, from complete virus production to partial expression of viral-specific proteins and/or RNA sequences, in different types of cell, strains of mice, physiological or experimental conditions. A number of genes controlling virus expression have been identified. A discussion of possible mechanisms controlling the expression of endogenous and exogenous viruses is given in a recent review by Weinberg and Steffen [22].

IB-2b. Type B RNA viruses. The type B viruses (for a review, see Bentvelzen and Hilgers [23]) are represented by the mouse mammary tumor viruses (Mu-MTV), isolated from the milk of C3H and A strains, which have a high incidence of mammary tumors. Endogenous Mu-MTV sequences have been found in all inbred laboratory strains tested (2 to 5 copies per haploid genome). Feral mice, however, proved to be free of Mu-MTV DNA. In contrast to type C viruses, which can be expressed in a variety of conditions, type B viral expression is limited mostly to the mammary gland in uninfected animals. It also occurs in other organs, but mostly in strains with a high incidence of mammary tumors or in exogenously infected mice [24], and the limited expression of endogenous sequences argues against a possible physiological role of the type B viruses.

IB-2c. Type A particles. Type A particles are of widespread occurrence in many murine tumors as well as in normal tissues [25]. Until recently, they had been considered as possible developmental forms of type B and C viruses. Although this may be true in some cases, the intracisternal type A particles (IAP) characterized by their localization in the cisternae of the endoplasmic reticulum have been shown, mostly through the work of Kuff and Lueders (see references mentioned below) to represent independent entities. Since, to our knowledge, no review article is yet available, we shall describe the IAP in some more detail. IAP have been purified from murine tumor cells, such as neuroblastomas and myelomas, and shown to have many properties in common with the type B and C viruses, in spite of their lack of infectious character. The particles contain a reverse transcriptase activity [26] and high molecular weight polyadenylated RNA [27], which can direct the synthesis of the 73 000 dalton major structural protein in a cell-free system [28]. These RNAs share no homology with those of type B and C viruses [29]. However, IAP share partial sequence homology with M432, a retrovirus endogenous to the Asian species *Mus cervicolor* [30]. By hybridization kinetics it has been shown that the haploid genome of laboratory mice, as well as wild mice, contains 500–1000 copies of sequences homologous to the RNA isolated from the IAP [31]. Studies on cloned DNA segments from these sequences have revealed that they are distributed throughout the mouse chromosome complement as homologous, but not strictly identical, units which, like type B and C proviruses, have terminal repeated sequences [32,33]. When cellular DNA is cut with restriction enzymes and analyzed by the Southern blotting technique, they show strikingly conserved banding patterns, not only within the various laboratory strains but also between these and feral mice. Kuff and Lueders, therefore, suggest that these genes may have evolved as endogenous cellular elements, and that some positive selective pressure may exist to maintain their organization. It is expected that it will soon be known if these repeated IAP genes present structural characteristics of bacterial-transposable elements, i.e., inverted repeats and flanking duplications of host sequences, as already shown for integrated avian and murine retrovirus proviruses [34–36]. More references on IAP and a discussion on the evolution of the sequences of this multigene family can be found in some recent papers [32,33].

IC. Background on early mouse embryogenesis

A brief account on early mouse embryogenesis is given here. The reader is referred to Theiler [37] and

Hogan [38] for further information on the early steps of mouse development. Many practical aspects of mouse embryo manipulation are dealt with in Daniel [39].

During the first 4 days of development (Fig. 3), the mouse embryo is free in the lumen of the oviduct and of the uterus, in contrast to later embryos which are implanted in the uterine wall. A distinction is made accordingly between preimplantation and postimplantation stages of development. 5–6 rounds of cell division take place during the preimplantation period, although not necessarily in a synchronous fashion. Until the 8-cell or early morula stage, all the blastomeres are totipotent, i.e., they are able to initiate cell lineages which can participate in the building up of any embryonic or extraembryonic tissue [40]. On days 2 and 3, the morula undergoes a process of morphological and biochemical differentiation which culminates in the formation of a blastocyst. This is a hollow sphere, the cavity of which is filled with liquid. It is composed of an outer layer of highly specialized differentiated tissue (the trophectoderm, also referred to as the trophoblast) and a small internal mass of about a dozen cells (the inner cell mass), which are still in an undifferentiated, pluripotent state. Up to this point, the embryo is

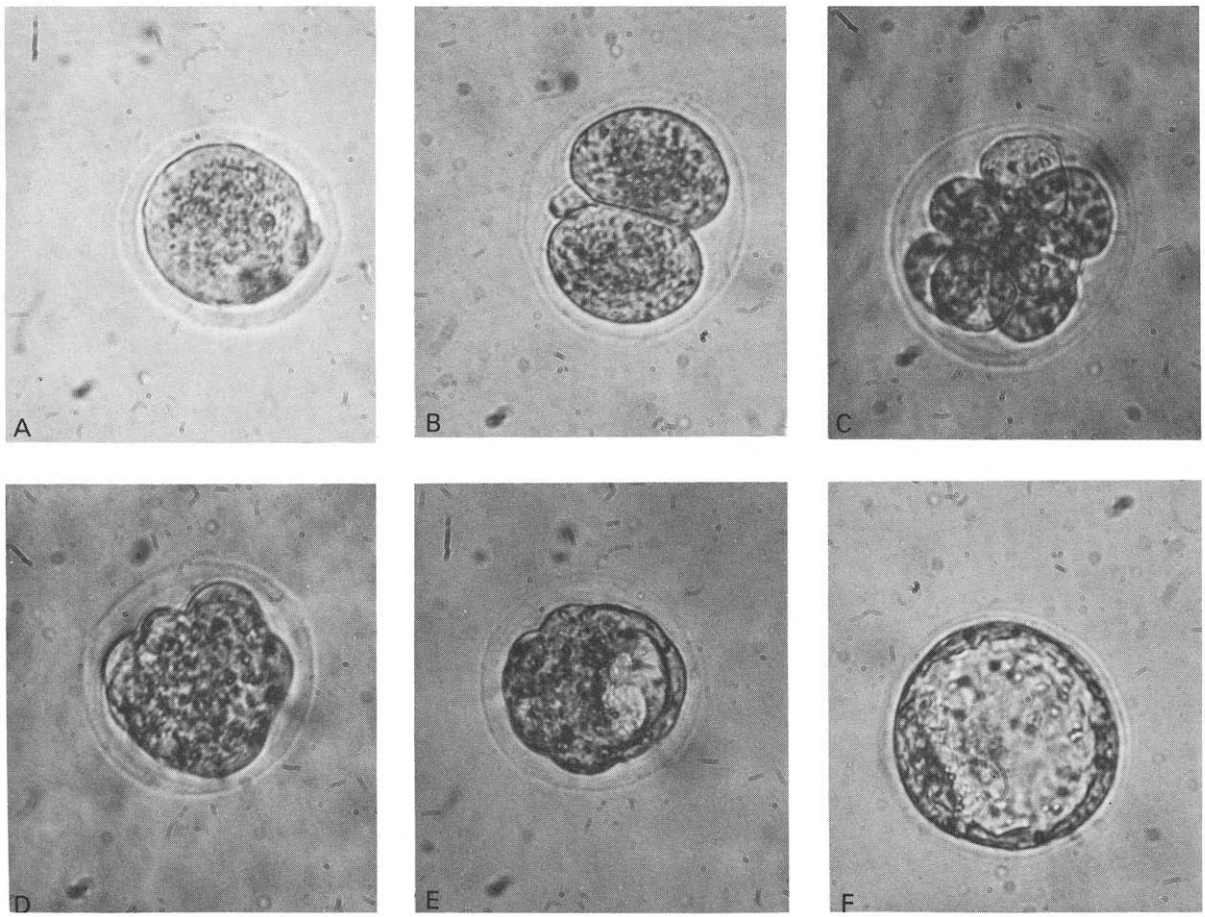

Fig. 3. Preimplantation stages of mouse embryogenesis. A: 1-cell egg (with one visible pronucleus). B: 2-cell stage. C: 8-cell stage. D: compacting morula (10–12 cells). E: early cavitating blastocyst. F: fully cavitated blastocyst (with the inner cell mass on the left). All the embryos are enclosed inside the zona pellucida. ×345. (Courtesy of Charles Babinet.)

enclosed in the zona pellucida, a thin membrane, which can be dissolved by pronase treatment. In vitro development of the embryo, with or without the zona pellucida, can be achieved from the 2-cell to the blastocyst stage with relative ease, using a simple, chemically defined medium. Blastocysts obtained in this way can be placed back inside the uterus of an adequately prepared foster mother. A high percentage of them will complete a normal post-implantation development.

After implantation (about day 4), growth of the inner cell mass results in the formation of an egg cylinder (Fig. 4A), enclosed inside an extraembryonic membrane, the yolk sac. The most important tissue in the egg cylinder is the embryonic ectoderm (or epiblast), a mass of actively proliferating cells [41], in which the proamniotic cavity progressively develops. Unlike any other cell in the embryo, epiblastic cells remain multipotent until day 7 of embryogenesis. An illustration of this property is the fact that, upon grafting to an ectopic site (e.g. beneath the testis capsule) the epiblast will give rise to a teratocarcinoma from which multidifferentiated tumors can be obtained [42,43]. On day 7, mesoderm cells are produced from the epiblast, and from this time on all the embryonic cell lineages are committed to some specialized developmental sequences. The only cells which remain totipotent are the germ cells. These are segregated from the somatic tissues around that time, and possibly 1 day later. On days 8 and 9 (Fig. 4, B and C), growth of the embryo is very rapid, and various processes of organogenesis are initiated, one of the most conspicuous being the sequential appearance of somites.

An essential feature of the early mouse embryo until day 7 is the persistence of an undifferentiated multipotent cell lineage. Embryonal carcinoma cells are the equivalent of this cell lineage. As such, they can give rise to a vast array of differentiated derivatives under various conditions [44,45], and may even participate in the building up of a normal embryo after they have been injected inside the cavity of a blastocyst [46].

II. Infection with viruses during early mouse embryogenesis

Gwatkin [47–51] appears to be the first to have studied the effect of viruses on cleaving mouse eggs in a systematic way. A variety of viruses (West Nile, Vaccinia, Rubella, Herpes Simplex, Cytomegalic Inclusion Disease, Polyoma and Mengo) were used to infect mouse preimplantation embryos at the 2-cell stage. Apart from Mengo virus, none of the viruses

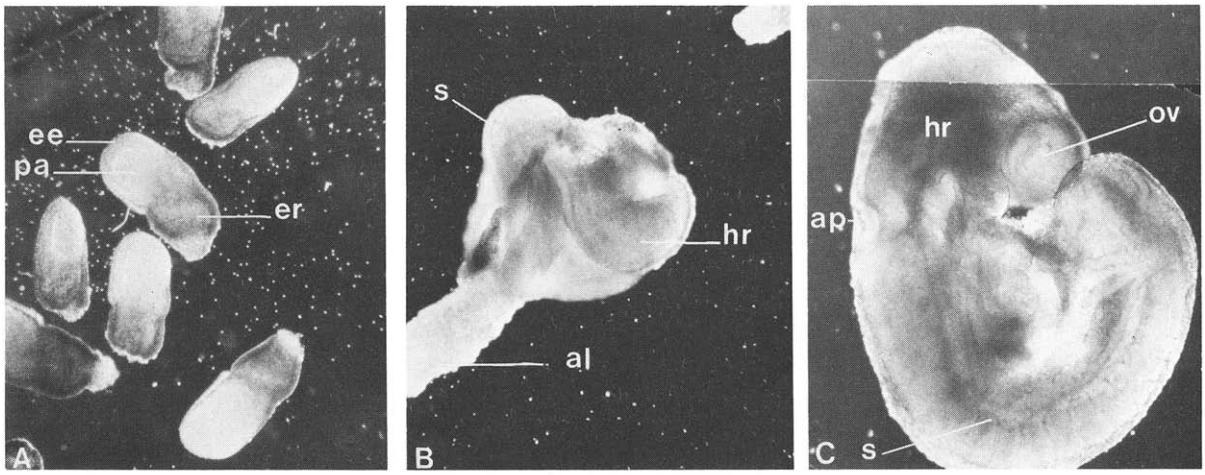

Fig. 4. 7–9-day postimplantation mouse embryos. A: 7-day embryos dissected from their trophoblast still surrounded by their yolk sac. ee, embryonic ectoderm; pa, proamniotic cavity; er, extraembryonic region. B: 8-day embryo dissected from its trophoblast, with a yolk sac partially removed. al, allantois; s, remnants of yolk sac. C: 9-day embryo free from its trophoblast and yolk sac. hr, head region; ap, auditory pit; ov, optic vesicle; s, somites. ×43.

tested produced any detectable change in the subsequent development of infected eggs, and there was no evidence of viral multiplication (polyoma virus-infected eggs were not checked for virus production). In contrast, 2-cell eggs infected with Mengo virus were blocked in their development. New viral particles were produced after a lag period about twice as long as in primary mouse embryonic fibroblasts. When embryos were infected at the blastocyst stage, the lag period was reduced to the value characteristic for embryonic fibroblasts. The reasons for this difference between 2-cell egg and blastocyst responses were not explored further, nor was the mechanism of the resistance of 2-cell embryos to infection by the other viruses.

During the past decade, investigations have focused on a few viruses. Two DNA tumor viruses (SV40 and, to a lesser extent, polyoma virus) and one RNA tumor virus (the Mo-MuLV) were used in fairly extensive studies and will be dealt with first. Information on two other viruses, adenovirus 5 and minute virus of mice (MVM), is also available and will be presented briefly later.

IIA. Infection with SV40

Mouse cells are not considered to be permissive for the replication of SV40 [13]. Upon infection with this virus, mouse fibroblasts will allow the expression of early viral functions, particularly the T protein, the intranuclear localization of which can be revealed in immunofluorescence tests. Expression of early viral functions may in turn result in the integration of viral DNA inside the genome of the host cell, which then acquires new properties characteristic of the so-called transformed state. It should be noted that no new viral particles are produced under these conditions, and that no cytopathic effect on the host cell is detectable.

The susceptibility of early mouse embryonic cells to SV40 was investigated first by Koprowski and his colleagues [52, 53]. Unfertilized mouse oocytes and preimplantation (2-cell and morula stages) embryos were placed for 1 h in a medium containing $4 \cdot 10^6$ plaque-forming units (PFU)/ml, followed by washing. Their subsequent in vitro development was observed for several days. It is legitimate to speak of the development of the unfertilized oocytes in this case, because they are subjected to the action of hyaluronidase, a treatment which triggers parthenogenetic development [54]. In no case was the development of early embryos affected by their being exposed to SV40 particles or DNA. However, when embryos taken at various preimplantation stages (2-cell, morula and blastocyst) were exposed to SV40 for longer periods of time (3 days, 2 days and 1 day, respectively) a different result was obtained [55]. The development of most 2-cell embryos was inhibited at a multiplicity of infection of $4 \cdot 10^3$ PFU per μl per embryo, while morulae and blastocysts did not stop growing until a multiplicity of infection of $4 \cdot 10^4$ PFU per μl and per embryo was reached. The presence of infectious viral particles inside or at the surface of the embryonic cells was revealed easily, 72 h after infection, by placing the infected embryo in contact with permissive monkey cells. A cytopathic effect on these cells was noted in all cases [53]. This was explored further in electron microscope studies of embryos which had been infected several days earlier at the 2-cell, blastocyst or egg-cylinder stage and subsequently grown in vitro [56,57]. Viral particles could be visualized on the surface of the cells or inside the cytoplasm. While nothing in these experiments proved that these particles were newly synthesized particles rather than persisting infecting viruses, various properties of certain cells were interpreted as indicating that actual viral replication occasionally did take place. These included alterations of nucleoli and chromatin, and were observed in rare endodermal cells of embryos raised in vitro from blastocyst or egg-cylinder stages.

The assumption that replication of SV40 does take place in at least some cells of the early mouse embryo leads to a paradox, since, as has been noted above (see above and Subsection IB), mouse cells are not thought to be permissive for this virus. In order to help clarify this point, SV40 DNA synthesis in early blastocysts was examined in an experiment by Jaenisch [58,59]. 4–8-cell embryos were infected with SV40 (10^7 PFU/ml for 4 h). 24 h later, the embryos, most of which had reached the blastocyst stage, were labelled with tritiated thymidine for 30 h. The radioactive DNA extracted from the embryos was hybridized to membrane filters loaded with SV40 DNA or mouse DNA. No significant hybridization to SV40 DNA was observed under these conditions. The

results were interpreted as suggesting that preimplantation mouse embryos are not permissive for SV40 DNA replication.

Evidence for the expression of early and late viral functions inside the trophectoderm cells of the mouse blastocyst, however, has been obtained more recently. Indirect immunofluorescence tests were performed on blastocysts which had been infected previously with SV40 at a multiplicity of infection of $4 \cdot 10^4$ PFU per μl and per embryo [55]. Both T and V antigens were detected inside the nucleus of some trophoblastic cells. The highest number of positive nuclei was seen when infected blastocysts were examined 48 h later. The number of V-positive cells per embryo was reduced about 10 times when the embryos were incubated in the presence of α-amanitin, an inhibitor of RNA polymerase II. This suggests that the immunofluorescence does indeed detect newly synthesized V antigen. Other points of interest are that in no case were the inner cell mass cells found to be positive for either T or V antigens, and attempts to demonstrate the appearance of new infectious viral particles under these conditions failed. The main conclusion one can draw tentatively from these experiments is that the first fully differentiated cells to appear in the mouse embryo, namely the trophoblast cells, allow the expression of both early and late functions of SV40, in contrast to the multipotent cells of the inner cell mass.

It is important to realize that the outcome of these experiments may depend very much on the conditions used for the infection of the embryos. Multiplicity of infection is clearly an important factor. Possible differences in the susceptibility of various mouse strains may also deserve further investigation.

The interpretation of the permissive nature of murine trophoblast cells for early and late SV40 functions remains, however, an open question. As far as the other cell compartments of the embryo are concerned, it seems that the block to the expression of early functions is not released before day 9, or possibly day 10, of embryogenesis. This is suggested by experiments in which post-implantation (6–10-day-old) embryos were dissected free of their trophoblast and yolk sac, partially dissociated, and infected with SV40 (10^8 PFU/ml) immediately or at various times after the cells had attached to tissue culture dishes. Indirect immunofluorescence tests for the presence of T antigen were performed 24–48 h after infection. Only rare positive cells were found in 6- and 7-day embryos, and in embryonic ectoderms from them, infected immediately or 24 h after dissection. In older embryos, as well as in later cultures of earlier embryos, an increase in the percentage of positive nuclei was observed, reaching 10–30% in 10-day embryos (Kelly and Condamine, unpublished data). The loss of their multipotent nature by epiblast cells, which occurs around day 8 of embryogenesis, does not seem to be correlated immediately with their becoming permissive for SV40 T antigen expression. A further delay of at least 24 h is necessary for this to happen.

The complexity of the relationship between SV40 and early mouse embryonic cells is exemplified further by experiments in which the exposure of early embryos to the virus results in the establishment of life-long relations between viral DNA and the host cell. Jaenisch and Mintz [60] showed several years ago that when mouse blastocysts are infected by microinjection of SV40 DNA into the blastocele cavity, development proceeds normally upon reimplantation of the blastocysts inside the uterus of a foster mother, and SV40-specific DNA sequences can be recovered from adult mice derived from the infected embryos. 10 animals out of 25 issue of such embryos proved to carry SV40 DNA sequences in their liver, kidney or brain cells. The number of copies of viral genome per mouse diploid genome, as determined by reassociation kinetics experiments, varied according to the tissue and the animal examined (0.5–3.6 copies in the liver and kidney tissues; 0.7–13 copies in the brain). Whether the viral genome was integrated inside the host cell DNA, or persisted as an independent plasmid-like structure, was not determined in this experiment, but the authors calculated that the amount of SV40 DNA recovered in the adult tissues corresponded to an increase in the initial input of at least two orders of magnitude. None of the animals which carried the SV40 sequences had any tumor at 1 year of age. No transmission of the viral sequences through the germ line was detected. More recently, Willison [61] has reproduced Jaenisch and Mintz's experiment, using the Southern transfer technique [62] to obtain new information on the state, free or integrated [63], in which SV40

DNA persists in the host cells of animals derived from infected embryos. The DNA of 14 newborn mice derived from blastocysts microinjected with SV40 DNA was analyzed in this way. Five animals proved to contain viral DNA, in a form undistinguishable from circular SV40 DNA (form II) as extracted from infected permissive monkey cells. Moreover, one male mouse contained viral sequences apparently integrated inside the host genome in both the germinal and somatic tissues. This male sired another male which contained the same arrangement of SV40 sequences, a strong confirmation that the virus DNA had been integrated in the DNA of at least some germ cells of the father.

In another series of experiments (Willison, Babinet, Boccara and Kelly, unpublished data), early morulae were infected with virus. When adult animals derived from these infected embryos were examined, no free virus could be detected in various tissues. Two out of ten mice, however, appeared to contain integrated SV40 DNA sequences, and in one of them the arrangement of sequences was the same in all organs tested, suggesting an early integration event. In addition, free viral DNA was found in some 12–14-day-old fetuses derived from infected embryos. In no case could SV40 T antigen be found either in cultures of midgestation embryos or in cultures from the skin of adult animals, nor did tumors develop. Whether the presence of free viral DNA results from a low level of productive infection of some cells or from the persistence of the viral DNA as an episome is at present not known. Thus, the available evidence suggests that the viral DNA can persist in a silent form inside the cells, with no damage to the embryonic development. Morulae infected with virus particles rather than with viral DNA appear to give essentially the same result.

In summary, there is no doubt that SV40 can be adsorbed on the surface of cells and penetrate inside them at any step of preimplantation mouse embryogenesis. While no viral function has ever been detected in multipotent cells following such penetration, evidence for the expression of T and V antigens inside the trophoblast cells can be obtained. Proof that viral DNA replication occurs under these conditions is lacking, however. Expression of early viral functions inside the embryo does not seem to take place on a very large scale before day 9 or 10 of embryogenesis. When viral DNA is introduced into morulae or inner cell mass cells, it can persist in at least some of the cell lineages descending from these cells for a long period of time (possibly during the whole life of the adult mouse) either in a free state or integrated inside the DNA of the host cell. It should be noted that in all cases where the virus DNA could be shown to have persisted during a long period of postnatal life, it was integrated inside the host DNA. Whether this is a necessary condition or not remains an open question. Transmission of the virus through the germ line may take place. At the same time, a new problem is raised concerning the mechanisms which ensure the persistence of viral DNA inside the embryonic cells for numerous cell generations, in what appears to be an essentially silent form. It may not be inappropriate to draw a parallel to the existence of cryptic DNA sequences, as have been detected in the progeny of abortively transformed cells ([64] and Fried, M., quoted in Ref. 13).

IIB. Infection with polyoma virus

Infection of mouse cells with polyoma virus is followed usually by a full cycle of viral replication, ending with the lysis of the cell (see Subsection IIA). A different situation appears to prevail, however, when the infection is carried out in preimplantation or early postimplantation embryonic stages [51]. No trace of viral adsorption at the surface or presence of the virus inside blastomeres of 2-cell stage embryos is detectable using electron microscopy techniques. In contrast, adsorption and endocytosis of the viral particles readily occurs in 3.5-day-old blastocyst cells and in 7.5-day-old egg cylinder cells. Various signs of viral replication can be detected in electron microscopy 4–5 days after infection, in some differentiated compartments of the embryos developed in vitro. These findings are compatible with unpublished data of Kelly and Condamine, who infected 7- and 8-day old egg cylinders with polyoma virus and looked for the production of viral antigens in immunofluorescence tests 48–72 h later. No V-positive cells were seen in such cultures. In contrast, V-positive cells were noticeable in cultures from 9-day embryos, and their frequency increased in cultures from 10-day embryos. As with SV40, there seems to be a lag period between the end of the multipotent state

and the time when the cells become permissive for polyoma virus replication. In the trophectoderm however, permissivity occurs as early as day 4, when this tissue has reached a differentiated state which sets it apart from the embryonic cells proper. This is shown by the results of immunofluorescence tests [55] in which V-positive cells are present among trophoblast cells of infected blastocysts, 1–2 days after infection.

The information presently available does suggest that the early multipotent cell lineages of mouse embryo are not permissive for polyoma virus replication, in spite of the fact that the virus is able to penetrate into the embryonic cells after the 2-cell stage.

IIC. Infection with Mo-MuLV

The interactions between murine leukemia viruses and mouse embryos have been studied in more detail than any other equivalent system. Much of the information presently available is due to the work of Jaenisch and his co-workers (see references below). Both exogenous (e.g., Mo-MuLV) and endogenous (e.g., AKR-MuLV) type C leukemia viruses are known in the mouse. AKR endogenous proviral sequences are transmitted vertically in a Mendelian fashion and their site of integration has been determined. Their activation and transcription in specific tissues results in the establishment of a viraemic state a few weeks after the birth of the animal, an event linked somehow to the appearance of a thymus-dependent leukemia after a few months (for a review of AKR leukemia, see Ref. 15). The experiments by Jaenisch and his collaborators demonstrate how an exogenous leukemia virus can be converted into an endogenous one with stable transmission, and how this conversion depends on the time at which the virus is allowed to interact with mouse embryonic cells. Apart from the light which these experiments shed on the relationship between the fate of the virus and the differentiation state of the cells which harbor it, they are important from an evolutionary point of view. They provide a model for the appearance of new endogenous viruses in natural populations, a phenomenon which has been postulated in various instances, by Todaro and his co-workers [65–67]. For the sake of clarity, we shall review separately the outcome of experiments in which mouse embryos were infected with Mo-MuLV at preimplantation and midgestation stages. Finally, the results of these experiments will be contrasted with those obtained by infecting stages close to birth.

IIC-1. Preimplantation embryos

4–8-cell Balb/c mouse embryos were infected with Mo-MuLV, either by placing them in a medium containing high (10^8 PFU/ml) amounts of virus [68], or by co-cultivating them with productively infected mouse cells [69]. (It must be noted that this is accomplished by treating the embryos with pronase, which removes their zona pellucida. Normally the embryo is deprived of this membrane for a short time only, i.e. prior to implantation as a blastocyst. This must be taken into account if the results obtained by Jaenisch are to be used for the interpretation of situations which occur in natural populations.) Yet another way of infecting the blastocyst in vitro is microinjection of one Mo-MuLV productive cell into the blastocyst cavity [70]. In vitro growth of the embryo is not affected essentially by the presence of the virus in the medium, and reimplantation into a foster mother of those embryos reaching the blastocyst stage yields a proportion of newborn animals comparable to uninfected controls. Blastocysts grown from infected 4–8-cell embryos were sectioned and examined by electron microscopy: no type C particles could be seen in their cells. X-C cell tests performed with these blastocysts in order to detect the production of new infectious particles were negative [68], as were immunofluorescence tests aiming at visualizing the presence of viral p30 and gp70 proteins [69]. It would seem then that under the conditions used by these authors, preimplantation mouse embryos are not infected productively with Mo-MuLV. Neither does exposure of 2-cell embryos or morulae to Moloney murine sarcoma virus (Mo-MSV) seem to affect their in vitro development to the blastocyst stage [53]. Infectious virus has been recovered from the treated embryos in this case, but it is not clear whether new viral synthesis has taken place or if the virus recovery is due merely to persistance of the infecting particles inside mouse embryonic cells for a few days after infection.

When mice born from Mo-MuLV-infected blastocysts are examined for the presence of virus in their serum, a variable number of them prove to be viraemic: 6.6% (three out of 45 animals) of mice from

embryos grown in a medium containing 10^8 PFU/ml [71] and about 40% when embryos were cocultivated with productively infected mouse cells [69,70]. Apparently, a much higher multiplicity of infection is constantly present in the latter case, which might account for the much more efficient results observed. Among the animals obtained from blastocysts microinjected with a Mo-MuLV-producing cell, about 10% appear to be viraemic [70].

In contrast to the apparent lack of interference of Mo-MuLV with mouse embryogenesis in these experiments, embryos growing in mice infected prior to or during gestation with Mo-MuLV [72], or with Rauscher murine leukemia virus (Ra-MuLV) [73] may be affected severely, as judged from histological examination, and substantial embryonic death may occur. In the case of Ra-MuLV, the earliest cytopathic effects reported were noted in blastocysts soon after implantation. Interestingly, after gastrulation, virally induced cytopathic effects are located essentially in mesoderm cells. These results, which differ from those obtained with in vitro infections, might be due to the presence of the virus at some critical stage of embryonic development after implantation. Such a critical stage should in any case be anterior to 8.5 day of gestation, since embryonic infection performed at that time does not interfere with the viability of the embryos [74]. An alternative explanation could be that deleterious effects are caused by virally induced disorders in the mother. The latter hypothesis is more likely, since there are strong indications that no transplacental passage of Mo-MuLV occurs during gestation [74].

Two mice, males Nos. 339 and 62, obtained from Mo-MuLV in vitro-infected Balb/c preimplantation embryos, and their progeny, have been subjected to detailed analysis in order to get quantitative and qualitative information about the presence of the viral genome inside their cells. A rather complex picture has emerged, which can be summed up as follows:

a. A small number (2–3) of Mo-MuLV-specific DNA copies were present in a great variety of tissues of male 339, including liver, kidney, lung, spleen, thymus, lymph nodes, testes (data from association kinetics experiments) [68].

b. Mo-MuLV-specific DNA was integrated inside host cell DNA sequences of both somatic and germinal tissues (data from DNA restriction enzyme analysis and progeny test experiments) [69,71,75].

c. Both males, 339 and 62, displayed mosaicism inside their somatic and germinal tissues, with some cells having Mo-MuLV-specific sequences integrated inside their genome, and other cells being free from viral sequences. Moreover, different germ cells could have the viral DNA integrated in different sites. When male 62 was mated with normal, virus-free females, viraemic and non-viraemic offspring segregated. Among the viraemic offspring, viral sequences integrated at different integration sites segregated in turn independently. Thus far, four different integration sites have been characterized by enzyme restriction analysis in the progeny of males 339 and 62. The corresponding genetic loci were named *Mov-1*, *Mov-2*, *Mov-3* and *Mov-4*. *Mov-1* was derived from male 339 and shown to be linked to *wa-1*, a locus on the mouse chromosome 6 [76]. *Mov-2*, *Mov-3* and *Mov-4* were all derived from male 62, and their mapping site is unknown [69]. In fact, *Mov-4* mice do not become viraemic (see below). Viral sequences at this locus have been detected by screening the non-viraemic progeny of male 62 by enzyme restriction analysis of liver biopsy DNA. Recently, the same test was applied to the non-viraemic progeny of another set of three mice (one male and two females), grown from infected preimplantation embryos. This revealed the existence of seven more integration sites (*Mov-5–Mov-8* and *Mov-10–Mov-12*) [70]. Furthermore, the viraemic progeny of yet another male has enabled the identification of an additional integration site, *Mov-9*.

The interpretation of these results is that when early mouse morulae or blastocysts are submitted to in vitro Mo-MuLV infection, each blastomere inside the embryo will have its own fate, irrespective of what happens to the surrounding cells. That is, integration of the viral genome may or may not occur, and when it does happen, it involves one (and sometimes two [69]) chromosomal sites out of several possible ones. 12 such sites are known at present, but it cannot be excluded that others will come to light in the future. Consequently, a mosaic embryo develops in which different cell lineages coexist, some of them carrying one or two virus-specific sequences integrated in possibly different sites, while others are free of any viral contamination. The ultimate,

remarkable effect of this is the transformation of an initially exogenous virus into an endogenous one, provided that some at least of the germ cell lineages have integrated the virus.

After a mosaic mouse obtained from an in vitro Mo-MuLV-infected morula has been bred, those segregants among the progeny which have inherited the viral DNA sequences will pass them on to subsequent generations in a Mendelian fashion [71], just as is the case for endogenous MuLV sequences in AKR mice [77,78]. In AKR mice, the generation of new endogenous virus-inducing loci has been observed repeatedly during the course of breeding programs aimed at transporting the AKR viral loci into virus-negative mouse strains [79]. It is very likely that these new loci arise as a result of proviral sequence insertions into as yet unoccupied chromosomal sites. Again, this suggests that the number of sites potentially available for the insertion of MuLV into the murine genome may be rather high. An important consequence is that it becomes possible to study the influence of DNA sequence environment on the expression of Mo-MuLV-integrated proviral genes. In fact, various mouse substrains obtained by integrating Mo-MuLV at the *Mov-1–Mov-12* loci have different patterns of viral production and leukemogenesis. Mice in which the proviral sequences are integrated at the *Mov-1* locus, either in homozygous or heterozygous condition [71,80], become viraemic early in their postnatal life (3–4 weeks) [70] and develop an acute thymus-dependent leukemia within a few months. A similar pattern seems to characterize animals which carry the *Mov-3* gene. In contrast, mice harboring the *Mov-2* gene become virus-positive only occasionally, and comparatively late in life (2–4 months). After they have become viraemic, *Mov-2* animals rapidly develop a leukemic syndrome essentially similar to that of *Mov-1* mice [69]. Finally, as noted above, animals carrying the *Mov-4–Mov-8* and *Mov-10–Mov-12* loci do not become viraemic. There are indications that the viral sequences integrated at *Mov-4* and *Mov-6* have been rearranged and possibly deleted at their 3′ end, which could account for the silence of the proviral genome throughout the life of *Mov-4* and *Mov-6* mice. No such rearrangement has been detected in the other non-viraemic substrains. It remains possible that their normal phenotype is due to small genomic modifications which go undetected in the enzyme restriction analysis performed at present [69,70].

The expression of viral RNA in various tissues of male 339 is documented in Ref. 68. Although the extensive mosaicism of this animal might have complicated the interpretation of the results, essentially the same pattern of expression was obtained for Balb/Mo mice, i.e. the mouse lineage obtained from male 339, which transmits the Mo-MuLV sequences integrated at the *Mov-1* locus in a strictly Mendelian way. Synthesis of Mo-MuLV-specific RNA occurs in spleen and thymus cells of Balb/Mo mice soon after birth, but not in liver, brain or kidney. A high level of expression is reached in lymphatic organs at 3–4 weeks of age and maintained thereafter throughout the life of the animals [70,81]. In the case of male 339, substantial expression of viral RNA in non-lymphatic organs (e.g. kidney) could be correlated with extensive infiltration of lymphoma cells inside such organs (by the time it was killed, the animal had developed a typical thymus-dependent leukemia). These results define an 'organ' or 'target tropism' [74] for the expression of Mo-MuLV sequences, which is restricted essentially to spleen and thymus cells. The same 'organ tropism' is seen in Balb/c mice infected shortly after birth, but not in mice derived from midgestation-infected embryos (see below).

IIC-2. Midgestation embryos

Microinjection techniques have been used to infect post-implantation mouse embryos with Mo-MuLV at day 8.5 or 9.5 of gestation by Jaenisch and his collaborators [70,74]. Although the surgery involved in this experiment appears to be rather sophisticated, more than 60% of the injected embryos survive to birth, no matter what material is injected, be it a viral suspension (about $5 \cdot 10^3$ PFU per embryo), or a control, virus-free medium. When tested at 4 weeks of age, about 50% of the animals derived from infected embryos prove to be viraemic. Their properties turn out to be rather different from those of animals obtained from embryos infected at preimplantation stages. Mo-MuLV-specific DNA sequences, as detected by association kinetics experiments, were found in all organs tested from viraemic animals. The list includes spleen, kidney, liver, brain, muscle, testis and lung. The number of copies per diploid mouse genome equivalent is frequently rather high:

up to seven copies in the kidney cells of some animals, always more than two and up to five copies in brain cells [74]. An even more striking result is obtained when tests are made for the expression of these sequences in different tissues. Viral RNA is present in large amounts not only in spleen cells, but also in kidney, liver, brain, muscle, testis and lung tissues. Some tissues contain greater amounts of viral RNA than the spleen cells, the only cells (in addition to thymus and lymph node cells) where viral transcription occurs when infection is carried out at preimplantation stages. Muscle tissue, for instance, can contain ten times as much viral RNA as spleen cells. Another peculiar feature of these viraemic mice is that they develop white hairs early in the post-natal life (i.e., around 6 weeks of age). Although the precise etiology of this phenomenon is unknown, it is tempting to follow Jaenisch's speculation [74] that it may be a consequence of viral expression inside melanoblastic cells. Together with the synthesis of viral RNA occurring in all tissues, this suggests that a regulation mechanism for the expression of viral functions quite different from that which prevails in Balb/Mo mice is at work in this case. Finally, a C57Bl/6 subline has been derived from a midgestation-infected male embryo in which the Mo-MuLV sequences had been integrated in the germ line at yet another locus (*Mov-13*). Germ line integration appears to be rare at that stage (one case in 250 mice tested). *Mov-13* mice have a very specific pattern of viral expression; i.e., the production of infectious particles is initiated during fetal life (probably around day 16 of gestation). Furthermore, in post-natal individuals, all tissues tested (muscle, kidney, brain, liver, heart, intestine and testis) appear to contain large amounts of viral RNA. An early hair color change is also seen in *Mov-13* mice. The occurrence of Mo-MuLV transcription in many (if not all) tissues of mice grown from midgestation-infected embryos has been documented in several animals. Such a generalized transcription has never been observed in individuals infected either at preimplantation stages or shortly after birth (see below), and it seems to be a characteristic feature of embryos infected at 8–9 days of gestation. It therefore requires a particular interpretation.

First, it is very clear that the differentiation state of a cell harboring integrated Mo-MuLV sequences in its genome is of prime importance for deciding whether viral transcription will occur or not. This point is of course illustrated by the restricted 'organ tropism' [74] of Mo-MuLV in mice derived from preimplantation-infected embryos. Furthermore, when Balb/Mo embryonic fibroblasts, which carry Mo-MuLV proviral sequences integrated at the *Mov-1* locus in a silent form, are fused with virus-free mouse thymocytes, expression of the viral p30 antigen occurs [82]. Fusion of Balb/Mo fibroblasts with macrophages or fibroblasts has no such effects. This suggests the existence of thymocyte-specific factors which control Mo-MuLV expression.

The differentiation stages reached by the cells of any organ in an adult mouse from a midgestation-infected embryo are identical, in all likelihood, to those of the same organs in a mouse derived from a preimplantation-infected embryo. Yet, the liver or brain cell proviral sequences will be expressed in the former case, not in the latter. Perhaps the site of provirus integration represents another crucial parameter in the modulation of proviral expression [70,74]. *Mov-13,* a locus apparently available for the integration of Mo-MuLV around day 8–9 of embryogenesis, has actually been shown to differ from the integration sites recovered from preimplantation embryos. More generally, the set of sites available for Mo-MuLV integration around day 8 might well be very different from those available around day 3. An observation made by Risser and Pollack [83] may be relevant in this context. These authors have shown that the ability of rat embryonic fibroblasts to undergo SV40 transformation varies considerably with their gestational age. The frequency of transformants peaks around day 16 of gestation, when it is about 100 times as high as at day 12. While the cellular mechanism responsible for this variation is unknown, it is conceivable that differential accessibility to integration sites is involved in this phenomenon.

Additional support to the concept of viral expression being governed by the chromosomal position at which the retrovirus is integrated comes from recent experiments in which cloned *Mov-3* DNA with its flanking sequences was injected into 1-cell mouse eggs, shortly after fertilization [84] (several viral and cellular genes have now been successfully transferred into developing mice after microinjection of various DNA recombinant molecules into the pro-

nuclei of fertilized mouse oocytes [85,242–246]). About 10% of the animals obtained from *Mov-3* DNA-injected embryos proved to be viraemic. One viraemic mouse (No. 158) has been studied in detail. One copy of the Mo-MuLV DNA per cell genome was present in all tissues of the animal. Enzyme restriction analysis showed that the flanking sequences of this newly integrated retroviral genome differ from those of *Mov-3*. The most likely interpretation is that the injected *Mov-3* DNA (but not its flanking sequences) has been replicated in embryonic cells prior to integration. Since integration at the same site is detected in all tissues, it must have taken place at a very early stage and viral DNA replication still earlier, possibly in the zygote itself (whether the hypomethylated state of the cloned viral DNA [86, 87] might have something to do with this replication remains an open question). The important point is that the pattern of Mo-MuLV expression in mouse 158, as revealed by the amounts of viral RNA measured in various tissues, differs from both *Mov-3* and *Mov-1* mice, again pointing to the prevalence of the chromosomal site of integration in governing this pattern.

It should be noted that this type of problem could be explored in other experimental systems as well. For instance, Franklin and Martin [88] have infected chicken embryos at the blastoderm stage by injecting avian osteopetrosis virus through the shell of newly laid eggs. After an eclipse phase when essentially no virus can be recovered from the embryo, viral production takes place in bones and lymphoid organs. It would be interesting to know if this 'organ tropism' could be modulated by changing the timing of infection.

Finally, another characteristic of midgestation-infected mice is the apparently low level of germ line integration. It is tempting to speculate that this phenomenon is related to the segregation of germ line cells from somatic tissues. It might be postulated that integration into the germ line is not possible after such segregation has occurred. In fact, germ line integration has never been detected in newborn MuLV-infected animals [70]. Since germ line segregation appears to take place in mouse embryos at about the time chosen for the midgestation infection experiments [89], germ line integration might be restricted to those embryos where segregation of the germinal tissue is lagging behind by chance.

IIC-3. Newborn mice

Although the experiments to be reported in this section do not deal with embryonic stages, they contribute further information upon the dependence of Mo-MuLV 'organ tropism' on the developmental stage at which infection takes place and therefore are of interest here.

When newborn Balb/c mice are injected with a low dose of Mo-MuLV (about 10 PFU per animal), a few of them will develop a thymus-dependent leukemia after a few months [68,75]. Mo-MuLV-specific DNA sequences are detected in thymus and spleen cells, but not in other tissues (liver, kidney, brain and muscle). (See below for discussion of possible productive infection of mammary gland.) Upon cleavage with *Eco*RI, the DNA from tumor cells (thymus or spleen) yields a rather complex set of fragments which hybridize with Mo-MuLV-specific sequences, just as the DNA from tumors of *Mov-1* mice. A $16 \cdot 10^6$ dalton fragment, however, which always characterizes the digestion profile of all *Mov-1* tissues (tumorous and non-tumorous) is not seen in the tumors from newborn infected mice. This implies that the *Mov-1* locus at which Mo-MuLV sequences can be integrated during early embryonic stages is no longer available for such integration in newborn mice, even in the target tissues (lymphatic cells), where the viral sequences are expressed [75]. This further exemplifies the notion that the integration sites are to some extent specific for developmental stages.

Apart from the thymus and spleen, the mammary gland may contain cells able to become productively infected with Mo-MuLV. This would explain the large number of viral particles present in milk from leukemic females [90]. The origin of these particles, however, is not known with certainty. They might be produced in lymphatic cells and transported from the serum to the milk [91].

Finally, it should be emphasized that there is no evidence that any productive infection or viral integration can occur in germ cells of perinatally infected mice [70,91]. Viraemic females will pass the virus to their progeny with a 100% efficiency when mated to normal males. In the reciprocal case, viraemic males do not transmit the virus. Conceivably, in females, viral transmission to embryos could occur in preimplantation stages (due to the presence of virus in the uterus). The presence of MuLV particles in

the uterine cavity of AKR mice has actually been documented by Rowe and Pincus [92]. Or it could be due to the passage of the virus through the placenta. Finally, transmission could happen after birth via the milk of viraemic mothers. The first two possibilities have been practically excluded by Jaenisch [74], who performed caesarean deliveries of viraemic mothers infected with Mo-MuLV shortly after birth. None of the 72 animals obtained in this way and subsequently fed by normal, non-viraemic mothers became viraemic. This is not to say that mouse placenta will always constitute such an efficient barrier to the passage of viruses from the mother to the offspring. Mouse cytomegalovirus, for example, does infect placenta cells when the mother is infected during gestation [93]. Although viral production does not seem to occur in this case, the presence of the virus in a latent form has been demonstrated recently inside embryonic cells [94].

IID. Infection with other viruses

Minute virus of mice (MVM), a non-oncogenic parvovirus [11], has been shown to infect 2-cell mouse embryos, provided their zona pellucida has been removed [95]. Preimplantation development is not affected by MVM infection, even though some virus production can be shown to take place at these stages.

The penetration of human adenovirus type 5 into the blastomeres of 8-cell mouse embryos is documented by an electron microscopy study [96,97]. There are indications that viral replication can take place shortly after penetration, which causes cell degeneration.

III. Endogenous viruses and their expression in mouse embryos

About 10 years ago, a possible role for endogenous retroviruses in normal embryonic development was postulated by Todaro and Huebner [98,99] and by Temin [102], in the context of the viral oncogene hypothesis and the protovirus hypothesis, respectively. According to the viral oncogene hypothesis [98,99], viral information for type C retroviruses is transmitted vertically in a partially or totally repressed state and behaves essentially as a cellular gene(s) under the control of cellular regulatory elements. Complete derepression of these endogenous viral genes in response to a variety of environmental or host genetic factors can lead to virus production and possibly to tumor formation. Partial expression of type C viruses can result in the production of virus particles and/or virus-specific antigens in the absence of a true transformed cell phenotype. Instances of such partial virus expression were recognized as early as 1970 [100,101]. They were at the origin of the suggestion that expression of viral functions is under the control of cell regulatory mechanisms and, somewhat more speculatively, that some at least of the functions of an endogenous virus might play a role also in specific developmental sequences. According to the protovirus hypothesis [102], the germ line contains potential genetic information (the protovirus) from which the actual information for malignant transformation can appear de novo in somatic cells. This is achieved by reverse transcription (i.e., information transfer from RNA to DNA) followed by insertion into new sites and introduction of somatic mutations, which allows new DNA sequences to be generated. In normal organisms, reverse transcription could play a role in the creation of new DNA sequences which might function during development in the establishment of successive differentiated states.

Although based on different premises, both theories have stimulated studies on the expression of virus functions in developing mouse embryos, all the more so as at the time these models were formulated, retroviruses represented one among few other possible tools to approach developmental processes at the molecular level.

In this section, experimental evidence concerning expression of endogenous viral genes at various developmental stages of the mouse embryo will be reviewed.

IIIA. Type C endogenous viruses in late mouse embryos

Some studies were done on mouse embryos during the second half of gestation, i.e. from day 10 to birth, and therefore will be mentioned here only briefly. Type C viral specific antigens have been detected with antisera against either the major structural glycopro-

tein, gp70, or the internal core protein, p30, using a variety of techniques, including complement fixation procedures [100], immunoassays [103] and immunofluorescence tests on serial sections of the embryos [104]. In these studies, viral specific antigens were observed on embryos from all laboratory strains of mice tested, as well as on feral mice, although quantitative differences were noted among different strains. Virus-specific RNA could also be detected in 14–18-day-old Balb/c mouse embryos [105]. However, no clear pattern of viral expression that could be correlated with a particular developmental process has emerged from these studies.

IIIB. Ultrastructural studies of type A and type C virus particles in early mouse embryos

Virus-like particles were first observed by Calarco and Brown [106] during a survey of the cytological and ultrastructural changes which characterize preimplantation development of the mouse. These particles were present transiently in all embryos examined. They appeared first at the 2-cell stage, were present in large number at the 4-cell and 8-cell stages, and only occasionally later at the morula and blastocyst stages. They were located within the endoplasmic reticulum sacs. In later studies [107–109], these particles were identified as intracisternal A particles (IAP). The same pattern of appearance of IAP, with a characteristic production peak around the 2- to 4-cell stages, was observed in embryos from numerous strains of mice. IAP are also found, although in small number, in the ovarian oocytes, until they are released from the ovarian follicles. They are not observed in the later phase of maturation of the oocytes, nor in the fertilized eggs [107, 110].

In a recent study by Yotsuyanagi and Szöllösi [110], a quantitative analysis confirmed the preponderance of IAP at early cleavage stages. In addition, the number of viral particles varies among different strains, being highest in AKR and NZB, intermediate in Balb/c, and relatively low in C3H, Swiss and DDK mice. These variations correlate well with the incidence of leukemia in these strains, with a 10-fold difference in particle number between the high and low leukemia strains.

Upon further analysis, the IAP found in early mouse embryos appear to fall into two distinct classes. Chase and Pikó [108] have distinguished between small IAP (abundant in the 2- to 4-cell stage embryos, declining from the 8-cell stage on, infrequent in blastocysts) and large IAP (always found in morulae and blastocysts, but rarely at the 8-cell stage, and never before). More recently, Yotsuyanagi and Szöllösi [110] further characterized the small A particles seen by Chase and Pikó by their morphology in electron microscopy. They present a distinct radial array of spokes between the envelope and the centrally located nucleoid (Fig. 5). This structure is reminiscent of R particles found in the hamster, as already noticed by Calarco and Szöllösi [107]. It is not found in IAP from a variety of tumor cell lines, including EC cell lines (Yotsuyanagi and Szöllösi, personal communication). These authors have proposed naming these particles 'ϵ particles' in view of their specific morphology and occurrence [110]. In spite of these structural differences, IAP from 2- to 8-cell embryos are stained specifically by an antibody raised against purified IAP from a plasmacytoma cell line [111]. Thus both types of particles share certain antigenic properties. There is also evidence that IAP isolated from different neoplastic cells may be different [112], although immunologically related through a common core protein antigen [113]. Whether IAP from different tissues are specified by different genes remains to be demonstrated. Due to the highly reiterated IAP genetic information in the mouse genome [31] and to the close homology of the related genes, as shown by cloning analysis, this may be a difficult question to answer [33].

In addition to large and small IAP, type C particles, characterized by a three-layered structure, have been found in early mouse embryos, but only at the blastocyst stage, and always in low numbers [108–110]. These are typical mature or immature type C particles (Fig. 5), either free in the extracellular space, or budding from the plasma membrane from both trophoblast and inner cell mass cells. Their presence has been detected in only some strains. For instance, they have been found in AKR [109], Swiss albino [108] and NZB [110] strains, but not in a number of other mouse strains, nor in feral mice [110].

A fourth type of virus particle was observed by Chase and Pikó [108] and its presence later confirmed by Yotsuyanagi and Szöllösi [110]. These are dense

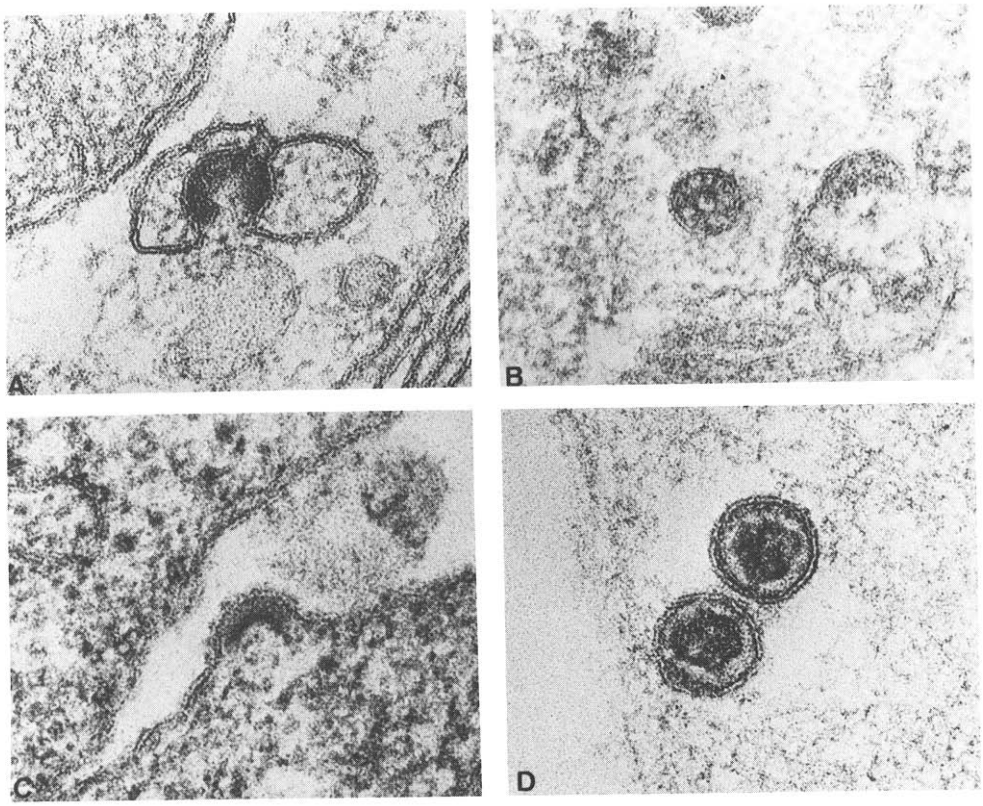

Fig. 5. Virus particles in early mouse embryos. A: Intracisternal A particles in early embryo of NZB mouse. ×113 000. B: Small intracisternal particle ('ϵ particle' [110]) in early embryo of AKR mouse. ×69 000. C: C-type particle budding at cell surface within a young blastocyst of NZB mouse. ×113 000. D: C-type particles within the zona pellucida surrounding a blastocyst of NZB mouse. ×113 000. (Courtesy of Y. Yotsuyanagi and D. Szöllösi.)

core vesicles about 50 μm in diameter, found free in the endoplasmic reticulum cisternae. While rare in cleavage stage embryos, these particles become more numerous in the blastocyst stage. Their nature and significance are not known at the present time.

So far, type B particles have never been reported, not even from strains with a high incidence of mammary tumor.

It should be noted that most of the studies reported above were carried out with embryos obtained from superovulated mice, but no difference was reported (except in the AKR strain, see below) between these and embryos at the same stage from spontaneous ovulation. It is unlikely therefore that the presence of particles could be attributed to the hormonal treatment. Likewise, when a comparison was made between in vitro cultured embryos and those obtained directly from the uterine horn [109,110], no difference was observed in virus expression. Finally, in embryos with parthenogenetic development, the distribution of IAP is comparable to that observed in normal embryos, ruling out the possibility that spermatozoa are necessary for the development of IAP subsequent to fertilization [114,115].

These results show that in normal development, IAP can be present in large quantities without apparent damage to the embryo. An unusually high number of IAP in blastomeres of preimplantation

TABLE I

DISTRIBUTION OF VIRUS-LIKE PARTICLES IN OVARIAN OOCYTES AND EARLY MOUSE EMBRYOS

Type of particle	Ovarian oocytes		Stage of embryo development						
	Dictyate	Metaphase II	Fertilized egg	2-cell	4-cell	8-cell	Morula	Blastocyst	7-day
Dense core particles	n.d. [a]	n.d.	0	±	±		±	++	n.d.
Small IAP	0	0	0	+++	++++	+++	+	+	n.d.
Large IAP	+	±	±	0	±	±	+	+	+
Type C	n.d.	0	0	0	0	0	0	+ [b]	+ [c]

[a] n.d., not described.
[b] Found only in AKR, NZB, Swiss albino strains.
[c] Found only in AKR strain.

embryos, however, is sometimes correlated with a pathological condition. A^y/A^y embryos, homozygous for the yellow allele at the agouti locus, die between early cleavage and implantation [116]. They can be recognized at the morula and blastocyst stage by the presence of large excluded blastomeres [117]. Ultrastructural studies [118,119] have shown a large number of IAP in these blastomeres, associated with other ultrastructural features characteristic of an earlier developmental stage, roughly equivalent to the 8-cell stage. The presence of numerous IAP in the excluded blastomeres thus may reflect arrest at an early developmental stage of embryos homozygous for the lethal gene A^y. In AKR embryos, abnormal blastomeres are often encountered at the morula or blastocyst stage. They have lost contact with the rest of the embryo and are filled with viral particles. These abnormal blastomeres are found only in embryos from superovulated AKR mice, suggesting that both hormone treatment and genotype are causally involved [120].

Very little information is at yet available concerning viral particles in early post-implantation embryos. The presence of IAP was reported in 7-day embryos from several mouse strains, whereas, at the same stage, type C particles were found in the AKR strain only [109,121].

These ultrastructural observations (Table I) suggest that the appearance and disappearance of viral particles is linked to specific developmental events. Large type A and type C particles are seen primarily at the morula and blastocyst stages, and the latter in certain mouse strains only. The production of small IAP at the 2- to 4-cell stage appears significant since the same pattern has been observed repeatedly in all strains of laboratory mice examined. In feral mice, in which the expression of endogenous retroviruses is very low [122], the same pattern of accumulation of IAP around the 2- to 4-cell stage has been observed, but no other particles are found later on [123,110].

Looking for a possible role for the small A particles, Chase and Pikó [108] have stressed the association of their production with the activation of nuclear and mitochondrial genes. In particular, from the 4-cell stage onwards, synthesis of ribosomal and transfer RNA is observed, rapidly followed by a massive production of ribosomes (for review, see Ref. 124). More recently, experiments by Johnson [125] have pinpointed the switching on of embryonal genes to the late 2-cell stage embryo, after DNA replication. However, the significance of IAP production at precisely this stage remains a question for which no real clue has emerged so far *.

* Somewhat puzzling is the situation observed in the Asian mouse species, *Mus cervicolor*. When early embryos of this species are examined in the electron microscope, IAP are found at the 8-cell stage, but not earlier [126]. *Mus cervicolor* contains a much smaller number of IAP-related genes (25–50 copies per haploid genome) [30] as compared to *Mus musculus* (500–1000 copies per haploid genome) [31]. It remains to be seen if the highly conserved amplification of IAP sequences in *Mus musculus* plays any specific biological role.

Recent studies have focussed on the structure of the IAP and the organization of IAP-related endogenous sequences in the mouse genome [32,33]. Further developments may open up new avenues for the understanding of these particles. In any case, molecular probes for the characterization of proteins and RNA species related to the IAP particles made in the early mouse embryo are now available.

IIIC. Antigens related to intracisternal type A particles (IAP)

The expression of IAP proteins has been studied by Huang and Calarco (Ref. 127, and personal communication). Using antisera raised against either IAP cores, which consist primarily of the major 73 000 dalton structural protein, or the purified protein itself, IAP-specific antigens could be detected on the surface of live 2- to 8-cell embryos by indirect immunofluorescence staining (Fig. 6). Zygotes showed low but detectable fluorescence, whereas unfertilized eggs, morulae, blastocysts and inner cell masses were negative. The kinetics of appearance are clearly different from those described for other surface antigens on preimplantation embryos [128–133], and coincide with the presence of IAP. Using the same IAP antiserum, a group of five proteins with molecular weights of 67 000, 69 000, 73 000, 75 000 and 77 000 could be immunoprecipitated from 2- to 8-cell embryos. The five bands were barely visible in the zygote, whereas morulae and blastocysts showed only the 75 000 and the 77 000 bands. The synthesis of the 73 000 dalton proteins correlates with the presence of IAP as seen in the electron microscope. It is thus likely that this protein is the major structural protein of the embryonic viral particles, but its relationship to the other proteins is not clear at the moment.

Two glycoproteins with a molecular weight between 65 000 and 70 000 had been detected previously on the surface of early embryos using mouse antiblastocyst rabbit antiserum. These components peak at the 8-cell to morula stage [132,133], that is, later than the IAP-related proteins. The possible identity of the 65 000–70 000 range proteins detected by the anti-IAP and antiblastocyst antisera awaits furhter demonstration. An interesting observation made by the authors is that, in contrast to early embryos, IAP-specific antigens are not detected on the surface of live cells of a rhabdomyosarcoma, although they can be demonstrated in their cytoplasm after fixation. This suggests the existence of a specific mechanism allowing the incorporation of IAP antigens into the surface of the embryonic cells. Further studies of the association of the 73 000 dalton protein with other embryonic proteins and of its relation with the cell surface may help to evaluate a possible role in surface recognition processes or surface-associated functions.

IIID. Antigens related to type C viruses in early mouse embryos

The information discussed above concerning stage-specific virus production during early mouse embryogenesis is compatible with the notion that proviral genes may be part of the animal genetic set-up during development [98,99].

A study by Pikó [134] illustrates a case in which a cellular gene, normally expressed in the mouse embryo at a very early stage, may have become incorporated inside the genome of a retrovirus. The experimental evidence includes the characterization, by means of immunocytochemical methods, of an intranuclear antigen, using a rabbit antiserum raised against purified p30 core protein from AKR-MuLV. This so-called germinal vesicle antigen is present in the oocyte during its terminal growth phase. It is detected in both male and female pronuclei after fertilization and in all nuclei of the cleaving embryo up to the 16-cell stage (Fig. 7). It is no longer seen in cavitating blastocysts. Five mouse strains examined by Pikó all appeared to possess this antigen, irrespective of their ability to produce AKR-type MuLV particles. Absorption experiments indicate that the germinal vesicle antigen shares antigenic determinants with viral products of AKR-MuLV and other, closely related viral strains. The normal function of the germinal vesicle antigen, however, is unknown at present, and there is no indication that the viral antigenic determinants which cross-react with it are involved in the oncogenicity of murine leukemia viruses.

In contrast, the direct relationship between normal cell genome components and viral genes presumed to play a key role in the establishment of carcinogenesis

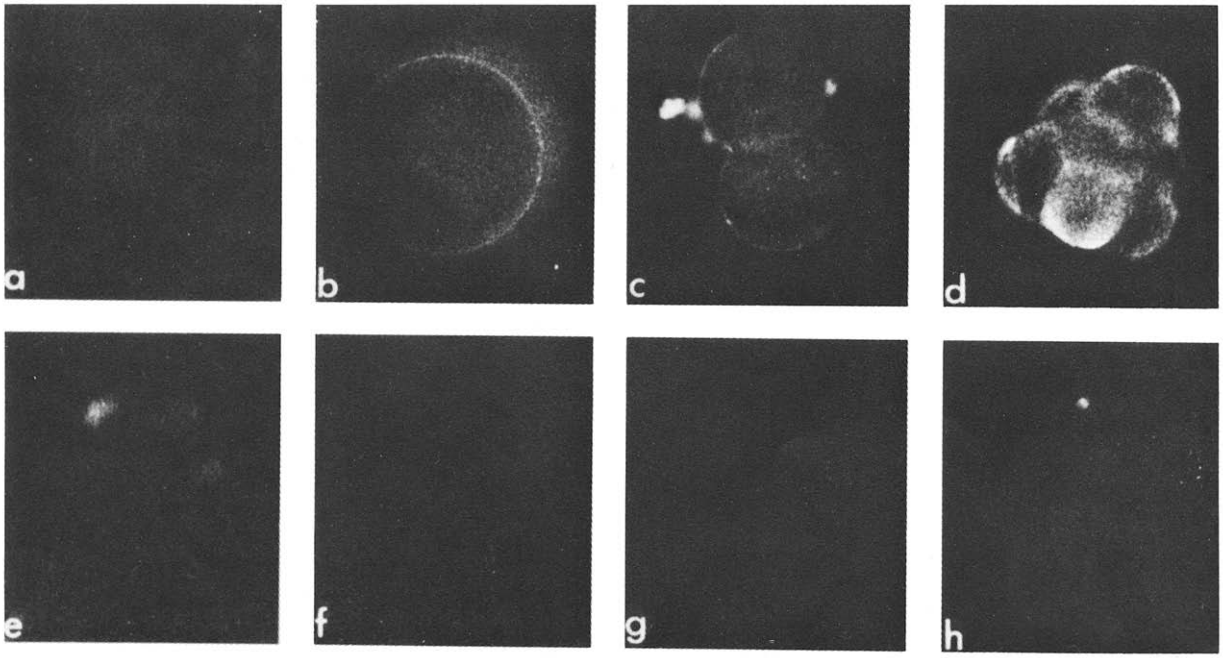

Fig. 6. Immunofluorescence staining of unfertilized eggs and mouse embryos with anti-IAP antiserum (Huang and Calarco [127]; with kind permission of authors and publishers). Anti-IAP: (a) unfertilized egg, (b) zygote, (c) 2-cell, (d) 8-cell, (e) morula, (f) blastocyst. Normal serum: (g) 2-cell and (h) 8-cell.

is a salient feature of recent models ([135]; other relevant references in this paper) which attempt to account for differences in transforming capabilities between acute and slow transforming RNA tumor viruses. According to such models, acute transforming viruses carry one normal cellular gene (*'c-onc'* gene) incorporated into their genome by some recombinational event. Once it becomes part of the viral genome, the *c-onc* gene is renamed a *'v-onc'* gene. The *c-onc* plays an essential role in the course of normal developmental sequences. Accordingly, it is expected to be transcribed, although at a comparatively low rate, in many normal tissues. The *v-onc* gene, on the other hand, has been put under the control of a viral promoter. No matter where a virus bearing such a *v-onc* integrates into the host genome, transcription of the *v-onc* gene will occur at a high rate (due to the characteristics of the viral promoter), resulting in the acute transforming phenotype of such a virus. In contrast, slow transforming viruses do not contain any *v-onc*. In order to transform a cell, they must integrate at one of possibly several specific sites, upstream and close enough to *c-onc* genes, so that the *c-onc* gene may again come under the control of a viral promoter, and be transcribed more actively. Several murine acute transforming retroviruses are known. All of them carry sequences derived from cellular sequences which are responsible for the acute transforming properties of the virus [136–139]. Information is available on the function performed by the transforming gene products of A-MuLV [140,141] and of Ha-MSV and Ki-MSV [142,143]. Yet virtually nothing is known about the functions that the cellular homologues of these transforming genes perform in the context of normal development, even for those which have been isolated (i.e. A-MuLV [137], Mo-MSV [138] and Ha-MSV [144]). In particular, it is not known whether these *c-onc* genes may play any role during embryogenesis, or if they merely are involved in fairly terminal differentiation processes [143]. It may be relevant to note that some *c-onc* genes represent very stable evolutionary units among vertebrate classes [145, 146], which may even be conserved among inverte-

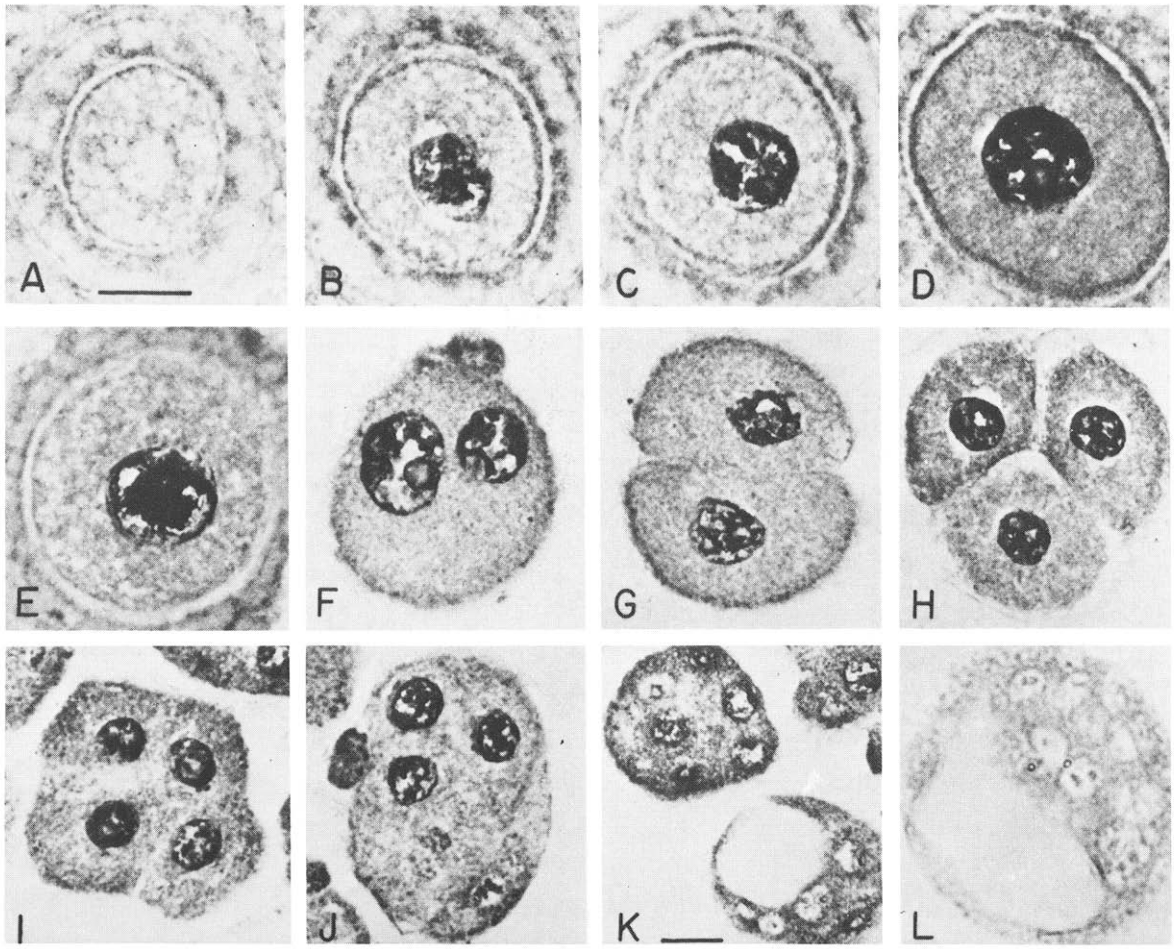

Fig. 7. Immunochemical detection of p30 MuLV-related germinal vesicle antigen in oocytes and early mouse embryos. A–D: Oocytes in 15-day-old ovaries with increasing staining reaction. E: preovulatory oocyte. F: 1-cell embryo with two distinct pronuclei. G to J: 2–16-cell stages. K and L: early and midblastocyst stages (from Pikó [134]; with kind permission of author and publishers).

brate groups [147]. In confirmation of this observation, the presence in normal cells of various vertebrate species of a protein closely related to Avian sarcoma virus-transforming protein has been documented [148]. Similarly, the protein coded for by the transforming gene of Harvey murine sarcoma virus (Ha-MSV) appears to have a normal cell counterpart which, using immunological and partial proteolytic peptide mapping, remains structurally very similar in cells of various mammalian and avian species [149]. If one accepts the speculation tracing back to Darwin (On the Origin of Species, 1st edn., pp. 439–450) that the earlier the stage at which developmental mechanisms act during embryogenesis, the more strongly these tend to be conserved during evolution, this observation might be taken as a clue that some *c-onc* genes are indeed involved in very early embryological steps. Such genes are detected after having been transposed into a proviral genome to which they confer high transforming properties. If proviruses integrate preferentially into transcriptionally active regions of chromosomes, as suggested by Breindl et al. [15], it is conceivable that screening viruses produced in mice obtained from early MuLV-

infected embryos (Subsection IIC) might provide a way to systematically detect early acting *c-onc* genes.

IV. Embryonal carcinoma cells (EC) and their interactions with oncogenic viruses

In early mouse embryos, the full virus growth cycle cannot be studied in detail. Biochemical characterization of the various steps is precluded by the limited amount of material. The detection of viral nuclear antigens by indirect immunofluorescence studies is hampered by the fragility of the embryonic cells when cultured in vitro and by their lack of adherence and rounded morphology.

In view of these technical problems, embryonal carcinoma (EC) cells derived from mouse teratocarcinomas provide an alternative. In many respects, EC cells resemble early mouse embryonic cells [38,45, 151,152] and can be grown in large quantities. They can be maintained in the undifferentiated state or induced to differentiate in vivo and in vitro into a variety of cell types, including derivatives of all three germ layers [153–157,247]. Their similarity to the undifferentiated cells of the normal embryo is emphasized in experiments demonstrating the ability of teratocarcinoma stem cells to be incorporated into the blastocyst and participate in normal embryonic development [46]. Conversely, EC cell lines can be derived from early embryos grown in vitro [158, 159]. The main characteristics of EC cell lines used in experiments to be reported below can be found in Table II.

IVA. Simian virus 40 and polyoma virus

IVA-1. Resistance to infection

The first study of the interaction between teratocarcinoma cells and DNA tumor viruses was carried out by Swartzendruber and Lehman [160] using populations of cells derived from the OTT6050 tumor, a transplantable teratocarcinoma isolated by Stevens in 129 strain [161]. These authors showed that when EC cells were infected with SV40 or polyoma virus, no T and V viral antigens could be detected by immunofluorescence staining. Increasing the multiplicity of infection to 1000 PFU per cell in

TABLE II

CHARACTERISTICS OF EC CELL LINES AND DIFFERENTIATED DERIVATIVES

For further information see Refs. 155, 247. 0TT6050: derived from a 6.5-day-old 129/Sv embryo grafted into the testis of a F1 (A/Hex129/Sv) host. 0TT5568: derived from a 3.5-day-old 129/Sv embryo grafted into the testis of a F1 (A/Hex129/Sv) host.

Cell line	Genetic background	Origin of the teratocarcinoma	State of differentiation	In vitro differentiation
F9	129/SV	0TT6050	EC	nullipotent
PCC4	129/SV	0TT6050	EC	pluripotent
PCC4a2 1	129/SV	0TT6050	EC	pluripotent
Nulli-SCC1	129	spontaneous	EC	nullipotent
SCC-S2	129SvS^J CP	0TT5568	EC	pluripotent
SSC-PSA4	129SvS^J CP	0TT5568	EC	pluripotent
PCC6	A/Heston	spontaneous	EC	nullipotent
PCC7-S	Recombinant inbred 129/C57B	spontaneous	EC	neural derivatives
PYS	129/SV	0TT6050	endodermal cells	
PCD1	129/SV	0TT6050	myocardial cells	
PCD2	129/SV	0TT6050	myoblast cells	
PCD3	129/SV	0TT6050	fibroblast cells	
F12	129/SV	0TT6050	unidentified differentiated state	

the case of SV40 did not result in the appearance of positive cells. In contrast, differentiated derivatives of EC cells were susceptible to infection, being permissive to polyoma virus replication and non-permissive for SV40. This was the case whether the differentiated derivatives were derived from spontaneously differentiated EC cultures, or from a line of parietal yolk sac (PYS) cells.

These results were confirmed on a variety of established cell lines. Two multipotent EC cell lines, PCC4-F and PCC3/A/1, derived from the same teratocarcinoma, were resistant to infection by SV40 and polyoma virus [162]. Three differentiated lines of fibroblastic (PCD3), myoblastic (PCD2) and myocardial (PCD1) cells were susceptible to lytic infection by polyoma virus and could be transformed abortively by SV40 [162]. Other EC cell lines derived from teratocarcinoma cells obtained from different inbred strains, C3H, C3HX129, A/Heston were also resistant to viral infection (Boccara and Kelly, unpublished data). In SV40-infected nullipotent F9 EC cells, even the more sensitive technique of immunoprecipitation followed by polyacrylamide gel electrophoresis did not allow detection of the two early SV40 gene products, large T and small t antigens [163]. Acquisition of susceptibility to viral infection was observed upon in vitro differentiation of several EC lines. PCC3/A/1 cells are able to differentiate in vitro in the course of about 3 weeks into many derivatives of the three embryonic layers [154,155, 247] and become susceptible to SV40 and polyoma virus infection, as shown by the progressive increase in the number of cells positive for viral antigens in immunofluorescence tests [162]. Viral products can also be detected when the morphological changes are triggered by a variety of chemical inducers, such as 5-bromodeoxyuridine (BrdUrd) [164], hexamethylene bisacetamide (Ref. 156 and Boccara and Kelly, unpublished data) and retinoic acid [165].

Taken together, these results indicate that a specific state of resistance to viral infection is associated with the undifferentiated state of EC cells.

The lack of expression of viral functions is not due to a defect of penetration of the virus into EC cells, as shown by several experiments. Swartzendruber et al. [166] used an approach which allowed detection of physical particles. SV40 virions labelled with [^{3}H]-thymidine adsorbed onto EC cells with kinetics comparable to those of differentiated cells. After cell fractionation, the majority of the radioactivity was detected first in the cytoplasm and later in the nucleus. Electron microscopy studies confirmed these results. Polyoma virus was found in the cytoplasm only and the reason for this difference is unknown. Another type of experiment aiming at following the fate of infectious particles – which in virus stocks commonly represent a minority of the total particles [13] – confirmed that they can enter EC cells. When EC cells infected with polyoma virus were fused to permissive differentiated cells, 20–30% of the heterokaryons expressed V antigen 48 h after infection, while homokaryons remained negative [167]. Finally, bypassing the adsorption and uncoating steps in virion processing by transfecting cells with SV40 or polyoma virus DNA bound to DEAE-dextran [166], or by microinjection (Boccara, Kelly and Graessman, unpublished data) also failed to induce T antigen. Thus, resistance of EC cells to infection with polyoma virus or SV40 appears to involve an early event in the viral cycle, after penetration and decapsidation, but before T antigen expression. In addition, the fusion experiments reported above indicate that, at least in the case of polyoma virus, susceptibility to infection in the differentiated cells is dominant over resistance in the EC cells. Cellular factors required for viral expression might be lacking in EC cells, and appear or be activated during differentiation [168].

Transcription studies suggest the existence of a defect in the production of normal viral mRNAs. When undifferentiated F9 cells were infected with SV40 and examined 10 h after infection – a time when SV40 RNA synthesis is at a maximum in permissive mouse fibroblasts – viral-specific RNA transcribed on both early and late DNA strands was found, although at a low level. In contrast to differentiated mouse embryo cells, which contain both normally processed early mRNA and non-spliced early SV40 transcripts, only the non-spliced species were present in F9 EC cells [163]. After treatment with retinoic acid, which induces cellular differentiation, stable, normally processed SV40 mRNA was produced and synthesis of early viral proteins could be demonstrated by immunoprecipitation and immunofluorescence tests [165].

It was suggested that the failure of RNA transcripts to mature into stable early mRNA might be due either to inefficient splicing mechanisms and/or viral RNA instability [163].

In the case of polyoma virus-infected PCC4/azaR1 EC cells there was no evidence for a block in RNA splicing. Low levels of viral specific RNA were detected 24 h after infection and the only species found were normally spliced mRNAs (Kelly and Kamen, unpublished data.)

IVA-2. Isolation of polyoma virus mutants capable of growth on EC cells

In another approach to understanding the nature of the block to polyoma virus replication in EC cells, mutants able to grow in these cells were isolated [169]. A first series of mutants was derived by selecting for viral ability to multiply on PCC4/azaR1 cells. Polyoma virus-infected PCCA/azaR1 cells were passaged every 2 days, until 2 months later 5–10% T antigen-positive cells appeared in the culture. The virus was recovered, plaque purified on secondary mouse embryo cells, and six virus clones, referred to as PyECPCC4, were isolated. When analyzed with restriction enzymes, each mutant exhibited an additional sequence of 20–50 base pairs located in the late region around position 0.69 close to the postulated origin of replication (Fig. 1). The selection procedure did not alter the main biological properties of the virus. In particular, oncogenicity, transforming ability, host range and burst size remained unchanged. Upon further analysis, two of the mutants were shown to present a sequence rearrangement consisting of a deletion and a sequence duplication ([170] and Fig. 8). Recombinant DNA molecules were constructed in which the rearranged sequence from the mutant replaces the corresponding region from an otherwise wild-type virus. The resulting virus was capable of infecting PCC4/azaR1, supporting the conclusion that the rearrangement observed in the 0.68–0.71 map units region is sufficient to account for the newly acquired viral host range.

Other polyoma mutants (PyECF9) have been selected by different authors [171–173] on similar basis for their ability to replicate in F9 cells. The PyECF9 mutants have their genome altered in the same region as the PyECPCC4 mutants, but changes appear to be less extensive. The minimal changes required for permitting expression on F9 cells involves the transition of a thymine into a cytosine and the insertion of 2 base pairs, 15 nucleotides apart ([171] and Fig. 8). Many of these mutants contain, in addition, small duplications of DNA segments in this region.

All mutants studied so far are located in the same region of the genome. This non-coding region is crucial for both DNA replication and initiation of transcription. It contains the postulated origin of replication, the sequences which encode the 5′-termini of early and late mRNAs, and a binding site specific for eucaryotic RNA polymerase II. It can be assumed, therefore, that a promoter for transcription of early mRNA is located in that region. A possible effect of the sequence alterations observed in the mutants might be to introduce new promoter sites that are functional in EC cells [169,170]. None of the mutants exhibits any detectable modification in the 0.78–0.86 map units region, which is involved in mRNA splicing. The hypothesis proposed by Segal et al. [163] that a defect in the splicing mechanism accounts for the resistance of F9 cells to SV40 would,

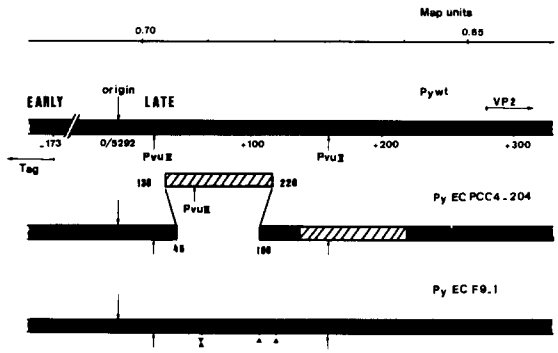

Fig. 8. Genomic changes around the origin of DNA replication in two polyoma virus EC mutants. The upper part of the diagram shows the organization of wild-type polyoma virus around the origin of DNA replication. The numbers correspond to the nucleotides, starting from the origin. The middle figure shows the rearrangements observed in the PyECPCC4-204 mutant: a deletion and the insertion at the deleted site of a duplicated segment of the Py genome. The duplicated and the inserted sequences are indicated with hatched lines. The bottom figure indicates the two insertions (▲) and the transition ⧗ in the PyECF9-1 mutant. The arrows (↓) and (↑) show the origin of replication and the PvuII restriction sites, respectively (adapted from Katinka et al. [170,171]).

therefore, not hold for polyoma virus. This is in agreement with the fact that no evidence for a block in polyoma virus splicing was found, as reported above. It cannot be excluded, however, that other events such as post-transcriptional mRNA processing or initiation of viral DNA replication may be affected in the mutants [172,173]. It should be noted that the rearrangements observed in the PyECPCC4 mutants do not allow growth in any EC cell line. They cannot, for instance, infect F9 cells. Likewise, PyECF9 mutants do not necessarily replicate on PCC4 cells [173]. The mechanisms involved in viral restriction for polyoma virus therefore might be different in both cell lines.

IVA-3. Persistence of functional viral DNA in infected cells

The fate of the infecting virus inside EC cells remains a question, independent of the mechanism of its restriction. It was investigated first in EC cells infected with polyoma virus which were then induced to differentiate under conditions where secondary infection was prevented [167]. A progressive increase in V antigen-positive cells was observed when the cells were induced to differentiate shortly after infection. In contrast, passaging the cells several times before the induction of differentiation prevented the appearance of positive cells. This suggests that, for a limited period, the virus is harbored in a silent state until the cells become competent for viral expression, but that it is diluted out rapidly along with cell division. More recently, it was shown that EC cells infected with SV40 still contain unintegrated full length viral genome 10 days after infection [174]. When the cells were induced to differentiate by treatment with *N,N*-dimethylacetamide, the cells retained SV40 in a non-integrated state but no T antigen could be detected. The viral DNA, however, was infectious upon transfection of permissive cells. These results suggest that SV40 genomes can persist in a silent form, as in the case of polyoma virus, but that the loss of the undifferentiated state per se is not sufficient for SV40 expression. A parallel can be drawn between this and results obtained with SV40-infected embryos (see Subsection IIA).

IVA-4. Expression of integrated SV40 DNA

Attempts to transform EC cells with SV40 by conventional methods have only resulted in differentiated cells expressing early SV40 antigens and transformed growth properties [175]. In order to examine the expression of integrated viral sequences in the context of EC cells, two techniques have been used. Somatic cell hybrids between PCC4/azaR1 cells and a BrdUrd-resistant clone of SVT2, an SV40-transformed mouse fibroblast cell line, were isolated, in the hope of obtaining a cell line with an EC phenotype and carrying an integrated SV40 genome [168,176]. Although most hybrids resemble the fibroblastic parent, a minority class show some of the properties of the EC parent (e.g. presence of specific embryonic antigens and absence of H-2) and is considered tentatively as an intermediate state of differentiation. Although expressing T antigen, hybrids from this class are less tumorigenic than the fibroblastic parent, and virus rescue by fusion with permissive cells is less efficient than in the transformed parent. This points to a regulation of viral expression by the state of differentiation of the host cell. More recently, Linnenbach and co-workers [177,178] succeeded in constructing a line of EC cells carrying a single integrate copy of the SV40 genome. This was accomplished by transfection of thymidine kinase (TK)-deficient F9 cells with a recombinant DNA molecule consisting of the plasmid pBR322 linked to the herpes simplex virus type-1 TK gene and to the genome of SV40 [177]. The clone 12-1 thus derived has antigenic properties of EC cells, as shown by the presence of the embryonic antigen SSEA-1 [178] and the absence of the major histocompatibility antigen, H-2 [131], and $\beta 2$ microglobulin and can differentiate into endoderm-like cells by treatment with retinoic acid [179]. While the TK gene is expressed in both undifferentiated and differentiated cells, SV40 early proteins can be detected, by immunofluorescence and immunoprecipitation, in the differentiated cells only [178]. The two, 2.9 and 2.6 kilobase, mRNAs normally found early after infection of permissive cells are present in both EC and differentiated cells in comparable amounts [180]. In contrast, transcription of the H-2 and $\beta 2$ microglobulin genes occurs in the differentiated cells only [179]. Different levels of control of gene expression are thus operating in this cell line.

IVB. Murine RNA tumor viruses

IVB-1. Resistance to exogenous MuLV

Several undifferentiated teratocarcinoma cell lines derived from the 129 strain have been shown to be refractory to productive infection by Mo-MuLV, as well as by several other MuLVs, including Gross-MuLV and F-MuLV [181,182]. This has been demonstrated by the negative results of various assays; formation of syncitial plaques in an infectious center XC assay, test for viral production in the culture supernatant by inoculation into susceptible mouse fibroblasts, and reverse transcriptase assay. When EC cells are allowed to differentiate in vitro [182], or when differentiated cell lines (PCD3 and PCD1) are used [181], productive infection occurs as efficiently as in 129 mouse embryo fibroblasts. Endodermal cells from PyS, an EC-derived cell line, or from in vitro differentiated stem cells remain resistant [182].

In contrast to all other mouse strains tested, no type C endogenous virus could be rescued from the 129 strain [183]. It could be imagined therefore that a more stringent regulation operating on endogenous viruses and specific for the 129 genotype might also interfere with productive infection of exogenous type C viruses in EC cells from this strain. This possibility appears unlikely, since PCC6, an EC cell line derived from the A/Heston strain, from which endogenous viruses can be induced, showed the same restriction to MuLV infection [184].

IVB-2. Early events following MuLV infection

VSV (MuLV) pseudotypes are virus particles which contain the vesicular stomatitis virus (VSV) genome packaged into an MuLV glycoprotein envelope. They can infect teratocarcinoma cells and embryo fibroblast cultures with the same efficiency, as measured by production of VSV (VSV is not restricted in EC cells, see below). This result indicates that receptors for MuLV are indeed present on the surface of EC cells. Therefore, a defect in adsorption and penetration of the virus appears unlikely. The same results and interpretation hold for endodermal cells [182].

The early events which follow adsorption and penetration of the virus in normally permissive mouse cells consists of the synthesis of various viral DNA intermediates initiated by the viral reverse transcriptase [185–187]. In PCC4/azaR1 cells infected with Gross-MuLV, the two major free intermediates, forms I and III, can indeed be characterized [188]. Their production peaks at 9 and 24 h after infection, respectively. The second burst of viral DNA synthesis, which is due, presumably, to secondary infection by virus progeny, is not seen in EC cells [188]. Infectious viral DNA can be extracted from EC cells 10 h after virus inoculation, showing that there is no obvious defect in the viral intermediates, but is no longer found 48 h after infection.

Conflicting results were obtained concerning the integration into the host genome of DNA sequences complementary to viral RNA. In reassociation kinetics experiments, integrated sequences were detected in one EC cell line (PSA4) infected by Mo-MuLV, but not in another line (S2) [182]. D'Auriol et al. [189], who used a different technique (agarose gel electrophoresis followed by transfer and hybridization), were unable to detect any integrated viral sequences in PCC4/azaR1 cells infected with Ra-MuLV. More recent experiments [190] provide indirect evidence for the persistence of Mo-MuLV sequences in infected EC cells. When PCC4/azaR1 cells are infected with Mo-MuLV, induced to differentiate in vitro by exposure to *N,N*-dimethylacetamide, and then treated with BrdUrd, virus is produced and can be identified as progeny of the original virus by peptide analysis of the viral p30 core protein. Persistence of the virus appears to be a rather frequent event, since about half the clones isolated from single infected cells are capable of producing virus upon differentiation followed by treatment with BrdUrd. Whether the viral information is integrated into the cellular genome or carried as an episome remains to be determined. Latent infection of EC cells may also occur accidentally, as suggested by the observations of Huebner et al. [191] who found DNA sequences homologous to AKR-MuLV in some EC tumor sublines passaged in vivo and derived from the original tumor OTT6050. Viral proteins and infectious MuLV could be detected after differentiation only. Furthermore, virus progeny can be obtained from Mo-MuLV-infected EC cells upon fusion with permissive cells [192], provided the fusion is performed 8–12 h after infection. So far, all attempts to demonstrate viral expression have been negative. Only level backgrounds of specific viral RNA can be detected in Mo-MuLV- [182] or Ra-

MuLV- [189] infected EC cells. Similarly, viral specific proteins (gp70 and p30) cannot be detected in EC cells infected with Mo-MuLV by immunofluorescence tests [190].

Taken together, these observations clearly indicate the possibility for MuLVs to persist in EC cells in a silent form.

It is conceivable that two different blocks are operating in EC cells. One would act early and be released by fusion with fibroblasts. The other would exert a later and more stringent control, which differentiation combined with treatment by halogenated derivatives (IdUrd or BrdUrd) could counteract. Finally, if MuLV sequences indeed integrate into the genome of EC cells (a point for which definite proof is still lacking), it would be interesting to identify the pattern of viral expression in chimeric mice obtained from blastocysts injected with such teratocarcinoma cells. This could shed further light on the regulation of viral expression in mice infected at preimplantation and midgestation stages (see Subsection IIC).

In any case, one should be aware that the results reported in this section were obtained with different EC cell lines, different virus strains, and somewhat different procedures of infection. Therefore, the possibility exists that the mechanism(s) responsible for resistance to infection by MuLV may not be strictly identical in all situations. Recent experiments by Levy and co-workers (unpublished data) show that it may be possible to infect EC cells under certain experimental conditions. For instance, PCC7-S EC cells can be infected productively with Mo-MuLV, provided a high multiplicity of infection (10 PFU/cell) is used. Cells from the PCC4/azaR1 line will not yield infectious virus until the multiplicity of infection reaches 100–1000 PFU per cell. Even at this multiplicity of infection, viral production is only temporary.

IVB-3. Expression of endogenous type B and C viruses

Limited information on the expression of endogenous viruses is as yet available. It was shown recently that the cellular DNA of mouse strain 129 contains multiple (8–10) copies of sequences related to the xenotropic endogenous type C viruses, as shown by reassociation kinetics using a complementary DNA probe prepared with a xenotropic virus from *Mus musculus* [193]. Only very low levels of specific viral RNA can be detected in both EC cells (PCC4), and differentiated cells (PCD1). This RNA, however, is polyadenylated and is processed in a way similar to that observed in chronically infected cells. Endogenous type B viruses behave somewhat differently. Mu-MTV-specific RNA can be detected in differentiated cells (PCD1 and PCD3) but not in EC cells ([194, and Crépin, unpublished data).

IVB-4. Summary and conclusions

Table III summarizes the data reported above, together with the results obtained with a number of other, mostly non-oncogenic RNA and DNA viruses. Salient characteristics of the viruses have also been included. Resistance to viral infection is clearly not a general property of EC cells, as these cells are able to support the growth of quite a number of viruses belonging to many different groups. Apart from mouse cytomegalovirus (MCMV), restriction after viral penetration has occurred is limited to viruses which belong to three different groups. The case of MCMV is ambiguous since no data are yet available concerning penetration into the host cell. The restricted viruses have in common their small size and the fact that they replicate in the nucleus. In addition, polyoma virus, SV40 and MuLVs are oncogenic. It is clear that none of these features alone (for example, oncogenicity) strictly correlates with restriction in EC cells. In all these cases, the block to viral infection lies at an early stage, since little, if any, transcription can be detected. (With MVM it is known only that no viral protein is made.) On the other hand, resistance of the host cell correlates well with its undifferentiated state.

It is impossible at present to decide whether the EC cells restriction mechanism is positive or negative, that is, whether it results from the presence of virus-inhibiting components or from the absence of components necessary for viral growth to take place. Nor can it be ascertained whether a single mechanism is at stake for the restriction of different viruses or if specific defenses are used against each virus. In connection with this, it should be noted that, while most cells will respond to treatment with interferon by the activation of a large spectrum of antiviral defense mechanisms, EC cells will exhibit only a more selective antiviral state. In interferon-treated EC cells, the growth of Sindbis virus and influenza virus is inhibited

TABLE III

SUSCEPTIBILITY OF EC CELLS TO INFECTION WITH VARIOUS VIRUSES, ALONG WITH SOME PROPERTIES OF THE VIRUSES

Information concerning the classification and general characteristics of the viruses are from Fenner et al. [11]. The nature of the genome is as fllows: type of nucleic acid (R = RNA, D = DNA); strandedness (1,2 = single-, double-stranded); shape (lin = linear, cir = circular).

Virus type	Virus group	Genome		Replication site	Onco-genicity	Replication in EC cells	Ref.
		Nature [b]	Molecular weight ($\cdot 10^6$)				
Minute virus of mice (MVM)	Parvovirus	D/1/lin	1.5–1.8	nucleus	–	–	195,196
Polyoma virus	Papovavirus	D/2/cir	3.5	nucleus	+	–	160,162
SV40	Papovavirus	D/2/cir	3.5	nucleus	+	–	160,162
Adenovirus type 2	Adenovirus	D/2/lin	23	nucleus	+	+	197
Vaccinia	Poxvirus	D/2/lin	160	cytoplasm	–	+	198
Herpes Simplex Type 1 (HSV-1)	Herpesvirus	D/2/lin	100	nucleus	?	+	199
Mouse Cytomegalo-virus (MVMV)	Herpesvirus	D/2/lin	100	nucleus	?	–	199
Coxackie B3	Enterovirus	R/1/lin	2.6	cytoplasm	–	+	199
Mengovirus	Cardiovirus	R/1/lin	2.6–2.8	cytoplasm	–	+	200,201
Encephalomyo-carditis (EMC)	Cardiovirus	R/1/lin	2.6–2.8	cytoplasm	–	+	201
Murine leukemia virus	Retrovirus	R/1/lin	3	nucleus	+	–	181,182
Lymphocyte chorio meningitis virus	Arenavirus	R/1/lin	3.5	cytoplasm	–	+	198
Pinchinde	Arenavirus	R/1/lin	3.5	cytoplasm	–	+	199
Vesicular stoma-titis virus (VSV)	Rhabdovirus	R/1/lin	4	cytoplasm	–	+	181,202
Sindbis virus	Alphavirus	R/1/lin	4	cytoplasm	–	+	199
Semliki forest virus	Alphavirus	R/1/lin	4	cytoplasm	–	+	199,203
Mouse hepatitis	Coronavirus	R/1/?	?	cytoplasm	–	+	199
Influenza	Orthomyxovirus	R/1/lin	5	nucleus and cytoplasm	–	+	201

[201], whereas vesicular stomatitis virus (VSV) [202], Mengovirus, encephalomyocarditis virus (EMV) [201] and Semliki forest virus [203] replicate equally in treated and untreated cells. Whether this is an indication of the existence of specific restriction mechanisms requires further exploration.

Polyoma EC mutants so far represent the more promising approach to an understanding of the molecular basis of this resistance. Similar approaches would be desirable with the other viruses, SV40 as well as MuLVs.

A broad survey of the available experimental evidence indicates that mouse embryos and the teratocarcinoma system resemble each other in their susceptibility to viral infection. In both cases, the undifferentiated cells (from morula, inner cell mass and embryonic ectoderm) are resistant to infection by SV40, polyoma virus and MuLV, whereas differentiated cells usually will express viral functions. A transitory period where most cells are morphologically differentiated and not yet susceptible to viral infection occurs in both cases. A closer examination of the correlation between cell types and susceptibility to viral infection by the different viruses might be valuable. This may now be possible since cytological markers which allow the monitoring of

the differentiation of EC cells are becoming available [204, 205]. Another approach is indicated by the possibility of isolating polyoma virus host range mutants on EC cells. Although it is not yet known if these mutants can infect the multipotent stem cells of the embryo, it is hoped that mutants from polyoma virus as well as from SV40 and MuLVs, capable of infecting and possibly transforming these cells, might be isolated.

V. Interactions between DNA tumor viruses and cell functions

Although the genome of DNA tumor viruses may become integrated into the host cell genome, a process which usually leads to the appearance of a transformed phenotype, vertical transmission of such viruses to the progeny of an infected host has never been observed (see, however, in Subsection IIA the preliminary evidence on this subject obtained by K. Willison). Consequently, no information is available about a possible orderly pattern of integrated DNA virus functions during normal embryogenesis. It does not follow, however, that one cannot detect any relationship between viral and cellular functions in this case. If DNA virus components are thought of as, at least partially, derived from normal cell components to which they remain more or less distantly related (a speculation which draws some support from the existence of normal cell DNA sequences homologous to viral sequences [206,207]), it might be possible to show that normal cell functions will fulfill some viral functions which have been inactivated, e.g., due to a mutational event. Conversely, cell mutants might in some cases be complemented by viral functions. Some evidence relating to this type of approach will be considered in the first part of this section. The induction of embryonic cell functions by viral infection or transformation will be reviewed next.

VA. Complementation between viral and embryonic cell functions

Host range mutants of polyoma virus have been isolated by Benjamin [208], who selected variants making plaques on polyoma-transformed but not on normal 3T3 mouse cells. Upon further investigation these host range transformation (*hr-t*) mutants proved to be complemented by a variety of other cells which do not necessarily contain any free or integrated viral sequences. The list includes type C RNA virus-transformed cells, SV40-transformed cells, primary baby mouse kidney cells and primary mouse embryo fibroblast [209]. 19 independently isolated mutants all belong to a single complementation group, suggesting that *hr-t* mutants inactivate a single viral function [210]. Their effect, however, is complex, abolishing both the ability to grow on 3T3 cells and to transform them. While primary mouse embryo fibroblasts or Mo-MuLV-infected cells appear to be permissive for *hr-t* mutant growth, they do not restore *hr-t* gene ability to induce a transformed state [211]. The exact nature of *hr-t* gene function is not known, but it can be understood, assuming that the *hr-t* gene product induces the appearance of two sets of cellular factors. One set will confer a permissive state for viral replication to the cell (this set would be constitutively expressed in primary embryo fibroblasts, and also be induced by Mo-MuLV infection). The other set of *hr-t*-induced cellular factors might be responsible for triggering a sequence of events leading to the transformed state [210]. According to this model, wild-type polyoma infection would lead to the reexpression of embryonic factors which are utilized for the benefit of viral replication. Such factors are present in mouse embryo fibroblasts. Information about their possible distribution in other embryonic compartments, particularly in early embryonic cells, is lacking. One obvious advantage of this model is that it readily accounts for the relation between two sets of cellular factors and one viral function. There is now evidence, however, that *hr-t* mutations are associated with the loss of at least two viral proteins. Lania et al. [212] have isolated rat cell lines in which the DNA of an *hr-t* mutant is integrated stably without conferring the transformed phenotype onto the host cell. Both middle T and small t antigens are absent from such cells, in contrast to large T antigen whose synthesis is detected readily. Furthermore, there are suggestions that the middle T protein is responsible for the transforming capacities of the virus [213]. Whether small t antigen is necessary for replication, alone or in combination with middle T, remains to be determined. In any case, primary mouse embryo fibroblasts appear to cure *hr-t* mutants

by providing a cellular ersatz for a viral function.

Attempts to complement embryonal cell defects with viral functions have been very limited so far. An example of such an attempt is provided by a study of t^{w18} mutant embryos [214]. t^{w18} is a complex mutation on mouse chromosome 17, and a member of a vast group of *t*-haplotypes, all of which have been isolated from wild mice (for reviews, see Refs. 215, 216). t^{w18} heterozygotes are normal, but homozygous t^{w18}/t^{w18} embryos die at around 8–9 days of gestation. Various defects related to the t^{w18} homozygous condition have been described. These point to mitotic disturbances in the embryonic ectoderm of preprimitive streak (6.5 days) embryos [217], abnormal mesoderm formation [218] or organogenesis [219]. The primary defect caused by the mutation remains unknown. 6.5–7.5-day homozygous embryos can be distinguished from their normal littermates by their morphology and by the use of karyotypic markers. Normal embryos provide actively growing primary cultures in vitro, while mutants grow very poorly, if at all. Upon transplantation under the testis capsule, 6–7-day homozygotes yield very few teratomas, and the rare teratomas thus obtained have very limited growth potentialities, in contrast to wild-type and heterozygous teratomas. SV40 transformation, however, allows the rescue of homozygous cells from such teratoma cultures. Permanent cell lines have thus been obtained which clearly appear to have a mesodermal origin since they differentiate in vitro into myotubes or adipocytes [214].

It is tempting to speculate that specific factor(s) might be missing in the t^{w18} embryos, at a particular time (7–8-day) and in certain cellular compartments (mesoderm cells). They could be activated (or their function could be replaced) by early SV40 products. Several experiments point to the possible role of SV40 early proteins in providing factors for cellular growth. For instance, SV40 infection can trigger the growth of cells arrested by serum starvation [220] and small t antigen has been shown to be necessary for the transformation of serum-deprived cells or contact-inhibited cells [221]. A comparison of the effect of both wild-type and SV40 mutants affected in either large T (tsA) or small t (dlF) antigens on the growth of t^{w18} homozygous embryonic cells should help to clarify this point. Preliminary experiments indicate that *dlF* mutants are deficient in transformation of these cells (Kelly and Condamine, unpublished data). It may be relevant to note in this context that small t mutants are unable to induce the appearance of centriole-related cellular proteins [222].

VB. Reexpression of embryonic cell protein in SV40-transformed cells

The fact that the tumorous cell state often implies the reexpression of embryonic or fetal cell functions has been emphasized many times (for a review, see Ref. 223). As a consequence, many studies have been devoted to the detection of tumor-specific transplantation antigens (TSTA) cross-reacting with embryonic or fetal cell antigens [224,225]. Along these lines, a wealth of information has been collected recently concerning a cellular protein present in embryonic cells and reexpressed in a variety of virus (particularly SV40)-transformed cells, as well as in chemically induced or spontaneous tumors.

When immune sera raised against SV40 tumors or against purified SV40 large T antigen are allowed to react with extracts from SV40-infected or -transformed cells, they yield a complex immunoprecipitate which, in addition to the viral large T protein, always proves to contain a protein species with a molecular weight in the range of 53000–56000 [226–231]. Partial peptide maps of p53* reveal that it is not related structurally to early SV40 proteins, so that coimmunoprecipitation of large T and p53 appears to be due primarily to an interaction between these two proteins [226]. Independent cell lines of a given mammalian species always have the same p53 structure, as judged from partial peptide maps. In contrast, different mammalian species (mouse, rat, hamster, monkey, man) have different p53s with various degrees of structural homologies. For instance, mouse and rat p53 proteins are more closely related than mouse and man p53 [234,235]; this strongly suggests that p53 is encoded in the cellular rather than in the viral genome. A definite proof of this

* Although several slightly different molecular weights have been reported for this protein, it will be attributed a 53000 M_r hereafter and referred to as p53 following Jay et al. [232]. p53 may in fact correspond to a family of closely related entities [233].

assertion is offered by the fact that p53 can be found in various cells free of any viral contamination, including embryonal carcinoma cells [227], primary cultures from midgestation (10–14 days) mouse embryos [236] and chemically induced sarcomas [237]. Furthermore, a number of cell lines transformed by RNA retrovirus or papovavirus different from SV40 have also been shown to produce large amounts of p53 [233,235]. Immunofluorescence tests performed with a monoclonal antibody to p53 detect the protein inside the cell nucleus exclusively, both in SV40-transformed and in virus-free cells [238]. Low levels of the p53 protein have also been detected in uninfected non-transformed cells [239, 235]. As far as SV40 is concerned, it appears that the large T protein is necessary for the triggering of increased p53 synthesis [239] and, possibly, for the maintenance of its induction and/or stabilization [236].

No definitive answer as to the normal function of p53 is yet available. The important observation has been made that this protein can both be phosphorylated [233,240] and act as a protein kinase [232]. Since numerous transforming proteins are associated with protein kinase activity (see discussion in Ref. 232), this is an indication that p53 might play a major role in SV40-induced transformation. It should not be viewed as an obligatory marker of the tumorigenic state, however, since it can be absent from highly tumorigenic cell lines [236] and present in rapidly dividing non-tumorigenic cells such as early passages of mouse 12-day embryonic cells and early passages of human kidney epithelium and fetal brain [232,236,240]. Rather than being indicative of an early embryonic state, p53 might be part of one among several other possible mechanisms which ensure a high mitotic activity. In the context of this review, it would be interesting to know whether p53 is present in mouse embryos before day 10 of gestation (the earliest step at which it has so far been detected), particularly in 6–7-day egg cylinder embryonic ectoderm, a tissue in which very high division rates are known to prevail [41].

VI. Conclusions

Several questions of major developmental interest emerge from the experiments reviewed here.

Small oncogenic viruses (SV40, polyoma virus) cannot express any function in early multipotent embryonic or EC cells. What is the nature of this block? How is the appearance of permissivity linked to the major cell determination events which occur in the 8–9-day embryo? The recently isolated mutants which bypass this block may bring decisive clues to answer these questions.

Viral genomes (Mo-MuLV and SV40), even though not expressed, can be integrated into early embryonic cell genomes. Is the mechanism of this integration different from the one at work in permissively infected cells? Are there specific time-dependent integration sites and, if so, how many?

When integrated into germ cells, Mo-MuLV (and SV40?) genomes become endogenous sequences vertically transmitted in new mouse sublines. How does the integration site influence the pattern of viral expression during embryogenesis and adult development? Further information on these questions may be crucial for the monitoring of experiments aiming at stable integration of foreign genes in the mouse genome [85,242–246].

Expression of endogenous retrovirus sequences is part of the developmental program of mouse embryo. What is its significance? To what extent can the study of viral functions provide insights into uncovering critical cell functions during embryogenesis?

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