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Murine Teratocarcinoma: A Model for Virus-Cell Interaction in a Differentiating Cell System

Thomas D. Friedrich and John M. Lehman

Department of Pathology, University of Colorado School of Medicine, Denver, Colorado 80262

The stem cell of the murine teratocarcinoma is refractory to infection with Simian virus 40 and polyoma. Utilizing various procedures, we attempted to alter this block to infection by modifying the infection procedure. Multiple infections with high-titer SV_{40} and pretreatment of cells with DEAE-dextran or the carcinogen 4-nitroquinoline 1-oxide did not induce embryonal carcinoma cells to produce T-antigen. Co-infection with adenovirus 5, which infects the embryonal carcinoma, and SV_{40} did not induce the expression of SV_{40} T antigen. Therefore, these procedures did not overcome the block to virus infection. The assay for the SV_{40} T antigen was immunofluorescence; however, the immunoprecipitation technique did not detect T antigen in the infected embryonal carcinoma cells. Finally, the viral DNA present in the embryonal carcinoma was examined for its ability to replicate. These studies showed that viral DNA was not replicating as assayed by the viral DNA's sensitivity to UV irradiation when replicating in the presence of 5-bromodeoxyuridine.

Key words: **murine teratocarcinoma, embryonal carcinoma, SV_{40}, infection, T antigen, immunoprecipitation, replication**

The murine teratocarcinoma is an interesting tumor model system since the stem cell of this tumor, embryonal carcinoma (EC), has the capability to differentiate into cells and tissues from all three embryonic germ layers [1, 2]. These differentiated cells can contribute to the development of a mouse, providing a model to define the molecular and cellular events involved in mammalian development [3, 4]. To allow further characterization of this tumor model, the embryonal carcinoma was adapted to tissue culture [5].

Dr. Friedrich is now at The Jackson Laboratory, Bar Harbor, ME 04609.

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With the establishment and characterization of the murine teratocarcinoma in tissue culture, it was feasible to attempt to assay expression of DNA, RNA, and protein as the stem cells differentiated. The SV_{40} virus was an ideal probe for studying the regulation of genetic information since a considerable amount of information was available concerning the expression of this virus in permissive and nonpermissive cells [6]. If differences existed between the stem cell and differentiated cell in regard to expression of the viral DNA, information might be obtained which would be useful in defining cellular controls. These studies showed that the EC cells were not infected with SV_{40} or polyoma virus as assayed by various procedures; however, the differentiated cells which are progeny of the EC cells were infected [7, 8]. Further studies showed that the block to virus infection was not at adsorption or penetration, and the inability to uncoat was not responsible for the block, since purified infectious viral DNA was not able to initiate T-antigen synthesis in EC cells, but was able to induce T antigen in the differentiated cell progeny [8]. Further, a possibility existed that interferon was responsible for this block; however, EC cells were shown to neither produce nor be protected by interferon, while the differentiated cells were protected and produced interferon [9]. Thus, interferon was expressed when the EC cells differentiated and, further, were not responsible for the viral block. These results demonstrated that the stem cell of the murine teratocarcinoma was "blocking" or modifying the expression of the SV_{40} genome, suggesting that a difference in gene expression (SV_{40}) existed between the undifferentiated and differentiated cells.

The studies in this paper attempt by various procedures to infect the stem cells with SV_{40} and detect the presence of the T antigen with the immunofluorescent and immunoprecipitation techniques. Further, a new procedure is utilized to determine whether the SV_{40} DNA is replicating in the EC cells.

MATERIALS AND METHODS

The teratocarcinoma cell lines utilized in this study included the $247DESCl_2$ line of embryonal carcinoma, which originally produced a variety of differentiated cells [5], and the PCC4azal cell line, a line of embryonal carcinoma which can be induced to differentiate with the addition of certain chemicals to the medium [10, 11]. Both EC cell lines were derived from the transplantable OTT6050 tumor of the 129 strain mouse. Mouse embryo fibroblasts were prepared from Swiss-Webster mice as previously described [12]. The cells, with the exception of PCC4azal cells, were grown in Eagle's minimal essential medium (Grand Island Biological Co.) and 5% fetal calf serum (FCS). The PCC4azal cells were grown in AutoPow (Flow Laboratories), also containing 5% FCS. The cells were subcultured with 0.25% trypsin in 0.1% EDTA in phosphate-buffered saline (PBS) with the exception of the $247DESCl_2$ cells, which were subcultured with 0.25% pancreatin (Grand Island Biological Co.) in PBS.

The SV_{40} used in these studies was the RH911 strain grown and plaque-assayed in CV-1 cells (American-Type Tissue Culture) [12]. This virus was stored at $-20°C$ and had a titer of 1×10^7 to 1×10^9 pfu/ml.

Immunoprecipitation was performed on cells washed 3X with PBS and refed with methionine-free medium containing ^{35}S-methionine (1,160 Ci/mmole) at 20 μCi/ml between 24 and 26 hours postinfection. After washing with cold TBS, the cells were removed with versene, pelleted, and lysed in 0.25 ml per 1×10^6 cells of 120 mM NaCl, 0.5% NP-40, and 50 mM Tris-HCl at pH 8.0 for 15 minutes. The extract was treated with 0.25 ml of 150 mM NaCl, 0.05% NP-40, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5, and normal sheep or hamster serum for one hour at 4°C. The activated Staphylococcus aureus [13] was added for 10 minutes to absorb immune complexes, and then following centrifugation, the supernatant was divided into two fractions to which either 10 μl of either normal or anti-SV_{40} T Syrian hamster virus was added [14] and reprecipitated with S. aureus [Robinson and Lehman, in preparation]. This material was centrifuged, washed, heated, and analyzed on a polyacrylamide gel according to Laemmli [15]. After electrophoresis, the gel was stained, destained, agitated in three volumes of "Enhance" (New England Nuclear), dried on filter paper, and placed on X-Omat R film (Kodak).

For the immunofluorescence assay, the cells were grown on glass 18-mm coverslips, fixed with cold (–20°C) acetone-methanol (70:30), and reacted with anti-SV_{40} T hamster antisera [14] by the indirect method [12]. The antisera to adenovirus 5 was kindly supplied by Dr. Louis Pizer. The cells were observed with a Leitz Ortholux microscope fitted with an HBO mercury vapor lamp and appropriate optics.

For the detection of SV_{40} viral DNA replication, the PCC4azal cells were infected with 50 pfu/cell of SV_{40}. The cells were incubated in the presence of 5 μg/ml of BUdR for 48 hours. This technique was used to detect the replication of SV_{40} DNA in CV-1 cells as previously described [16]. For these studies, the nuclear DNA was extracted from the BUdR and non-BUdR-labeled cells and irradiated in 0.1 M Tris-HCl, pH 7.0 (2.4×10^4 erg/mm^2 of UV). The DNA was cleaved with XbaI, which does not cleave the SV_{40} DNA. Then, the DNA was electrophoresed in 0.8% agarose, transferred to DBM paper, hybridized with ^{32}P-nick translated SV_{40} DNA [17] and exposed to X-Omat R film at –70°C using an intensifying screen [18]. An increase in the component II DNA would suggest an increase in sensitivity to UV, indicating incorporation of BUdR into the replicating DNA.

RESULTS

The immunofluorescent procedure is able to detect small numbers of cells producing T antigen, but may be incapable of detecting cells producing low levels of T antigen per cell. Since the immunoprecipitation procedure may be more sensitive in detecting low levels of T antigen per cell, this technique was employed to demonstrate T antigen in SV_{40}-infected 247DESCl$_2$ cells. Figure 1 shows that SV_{40}-infected mouse embryo cells contain a 100,000-dalton protein precipitated by the anti-T serum, but not normal serum. This protein was not visible in the EC-infected cells even with long exposure times. A similar result was found with the PCC4azal cell line following infection with SV_{40}. Using a slightly different procedure – a one-hour methionine starvation followed by a one-hour methionine pulse (100 μCi/ml) – SV_{40}-infected mouse embryo and PCC4azal EC cells were examined for the 17,000-dalton small t antigen. No small t antigen was detected in the EC cells. A two-week exposure to x-ray film still failed to show any small t in the SV_{40}-infected EC cells.

Various attempts were made to stimulate T-antigen production in EC cells by using altered infection techniques. Several of these techniques will be briefly listed; however, all attempts were negative in their ability to stimulate T-antigen induction which was assayed by immunofluorescence.

Multiple infections were performed on the 247DESCl$_2$ with different pools of non-plaque-purified SV_{40} every 24 hours for three days. The titers of the pools ranged from 2×10^7 to 6×10^7 pfu/ml, and the cells were infected with approximately 100 pfu/cell. With this procedure, it was hoped to increase the levels of intracellular virus or that com-

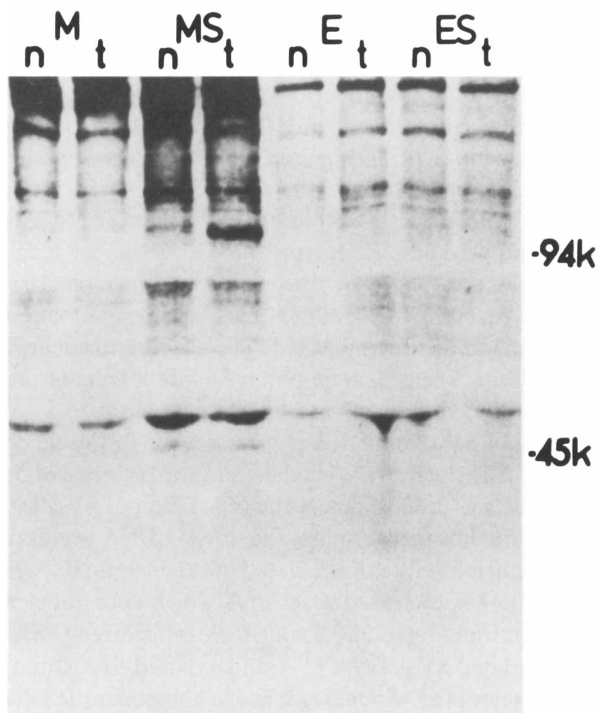

Fig. 1. Immunoprecipitation of T antigen in mouse embryo and embryonal carcinoma cells. Autoradiogram of electrophoretic pattern of immunoprecipitates. Mouse embryo cells (M) and 247DESCl$_2$ (E) were infected with SV$_{40}$ (MS and ES). The ^{35}S-labeled cell extracts were precipitated with either normal serum (n) or hamster anti-SV$_{40}$ T antisera (t). The amount of protein in each track comes from an equal number of cells. The mouse embryo tracks contain twice the number of counts as the embryonal carcinoma tracks.

plementation between the various pools would overcome the block. The EC cells were T antigen negative every day for seven days, while mouse embryo fibroblasts were greater than 90% T antigen positive by day 7.

DEAE-dextran has been reported to increase virus uptake 13-fold [19]. When 247-DESCl$_2$ were infected with 200 pfu/cell of plaque-purified virus in the presence of 100 μg/ml of DEAE-dextran for 45 minutes, no T antigen was detected at 24 and 48 hours post-infection. However, certain differentiated teratocarcinoma cells which were refractory to SV$_{40}$ infection were observed to induce several T-antigen positive cells in the presence of DEAE-dextran.

Pretreatment with the carcinogen 4-nitroquinoline 1-oxide (4NQ0) has been shown to increase the amount of integrated SV$_{40}$ DNA and transformation in SV$_{40}$-infected Chinese hamster cells [20]. This increase in integration may be related to the increased strand breakage of DNA, increased virus uptake due to membrane alterations, or possibly other reasons. When 247DESCl$_2$ cells were grown in 0.4 μg/ml of 4NQ0 for 24 hours prior to infection with 200 pfu/cell of plaque-purified SV$_{40}$, then cultured in the presence of 4NQ0, the cells failed to express T antigen up to 72 hours postinfection.

Adenovirus 2 has been demonstrated to replicate in EC cells [21]. When the 247-$DESCl_2$ line was infected with 50 pfu/cell of adenovirus 5, a small percentage of EC were induced to synthesize adenovirus V antigen as assayed by immunofluorescence. To determine whether adenovirus could "help" SV_{40} express T antigen, EC cells were co-infected with both viruses, but again, no SV_{40} T antigen was expressed by this modified infection procedure up to 72 hours postinfection; however, a small percentage of cells expressed adenovirus 5 V antigen.

In a recent publication, SV_{40} DNA was shown to be present in the EC nucleus for long periods of time before it was lost without integrating into the cellular genome [22]. Since this viral DNA was present in the EC cells, it was necessary to define whether this viral DNA was replicating. This was assayed with a protocol which detected supercoiled SV_{40} DNA replication in CV-1 cells in the presence of BUdR [16]. The BUdR-labeled supercoiled DNA molecules were detected by their susceptibility to nicking when exposed to short-wave UV light. The nicked molecules (form II) were separated from the unnicked molecules (form I) with agarose gel electrophoresis and detected by Southern gel analysis with nicked translated SV_{40}-labeled DNA. Figure 2 shows that there is no visible shift from form I to form II DNA in the BUdR-containing UV-irradiated samples. Therefore, the form I DNA present in the PCC4azal cell nuclei was not replicating (incorporating BUdR), which complements other data [22].

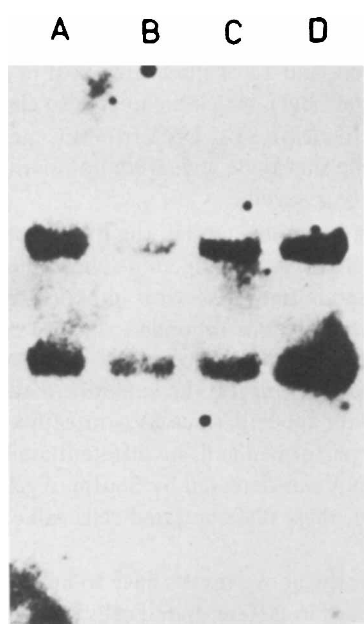

Fig. 2. Replication of SV_{40} form I DNA in PCC4azal cells. All tracks contain 10 μg of nuclear DNA cleaved with Xba I transferred to DBM paper and hybridized with nicked translated SV_{40} DNA. A) No BUdR, no UV; B) BUdR, no UV; C) no BUdR, 24×10^3 erg/mm^2 short-wave UV; D) BUdR, 24×10^3 erg/mm^2 short-wave UV.

DISCUSSION

Embryonal carcinoma cells infected with SV_{40} virus were not detected by the immunoprecipitation assay to contain the viral-specific T antigen. This technique was utilized since it is a sensitive assay and would detect levels of T antigen that may not be detectable by the immunofluorescence technique (per cell). Various procedures were attempted to enhance the uptake of SV_{40} virus and possibly modify the cells' capability to be infected with the virus. However, all of these treatments were negative. In one further series of experiments, a new procedure was utilized to detect the presence of replicating SV_{40} DNA in the PCC4azal line of embryonal carcinoma. The results of this study showed no detectable viral DNA synthesis. This procedure has been utilized to detect SV_{40} DNA replication in CV-1 cells [16]; however, it is conceivable that this procedure may not be able to detect a low level of viral DNA synthesis. These results, however, are compatible with our previous findings concerning the fate of the viral DNA in EC nuclei [22]. With various multiplicities of infection, SV_{40} viral DNA was detected by Southern gel analysis up to two weeks, but not at five weeks postinfection. This viral DNA could be recovered from the nuclei and shown with transfection studies on CV-1 cells to initiate infection (T antigen, viral DNA synthesis, V antigen, and infectious virus) [22]. The amount of viral DNA decreased with time and was not found with Cot reannealing analysis to integrate into the EC cell DNA [22]. Therefore, the viral DNA was present for a long period of time, did not integrate into the cell DNA, did not replicate, and eventually was diluted out, possibly through cell division.

Methylation of cytosine in the dinucleotide CG has been demonstrated in certain systems to modify gene expression, and 27 of these sites exist in the SV_{40} genome. When three restriction enzymes (HhaI, HpaII, BglI), which are unable to cleave if the DNA was methylated in these sites, were used to cleave SV_{40} DNA from EC nuclei, the patterns of cleavage were unaltered [22], suggesting that these sites were not methylated. However, the other methylation sites should also be assayed.

SV_{40} messenger RNA has been detected in the F9 line of EC following infection with SV_{40} virus; however, the RNA was a long, non-spliced message which led the authors to conclude that the inability to initiate SV_{40} virus infection may be due to the post-transcriptional modification of the RNA, possibly due to lack of certain splicing enzymes [23, 24]. If this was the mechanism, then the viral DNA present in EC nuclei which were induced to differentiate should then express the viral information. The differentiated cells contain the enzyme necessary for splicing, since SV_{40} infection proceeds normally in these cells. These experiments were performed and the differentiated cells did not express the T antigen even though viral DNA was detected by Southern gel analysis and transfection onto CV-1 cells [22]. However, these differentiated cells will express the SV_{40} T antigen when infected with SV_{40}.

Possible explanations for the above results have to include the following facts: 1) The viral genome is not expressed in differentiated cells obtained from SV_{40}-infected EC cells; 2) the SV_{40} DNA may be modified but when transfected into CV-1 cells, the SV_{40} DNA isolated from the EC cells can transcribe and translate; and 3) the viral mRNA is not spliced in EC cells. These facts may be explained by the presence of a modification to the DNA, nature unknown, that may allow some mRNA transcription but may inhibit splicing of the mRNA which is either made at a low level or is more susceptible to degradation.

This modification persists even though the cells differentiate, but is removed when the viral DNA is extracted from the EC cells, or possibly that the permissive monkey cells (CV-1) are able to specifically bypass this modification.

These studies have demonstrated that the murine teratocarcinoma is an ideal system for comparisons of gene regulation in a differentiating cell system. When the mechanism responsible for the SV_{40} block is understood, this may suggest possible mechanisms for cellular gene regulation.

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