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Carcinogen-Mediated Amplification of Specific DNA Sequences

Sara Lavi

Department of Virology, Weizmann Institute of Science, Rehovot, Israel

A model experimental system based on SV40-transformed Chinese hamster embryo cells and a highly sensitive in situ hybridization procedure was designed. Exposure of the cells to different categories of chemical and physical carcinogens resulted in the induction of SV40 DNA synthesis in the treated cells. Although the carcinogen-mediated amplification of SV40 DNA sequences is regulated by the viral "A" gene, neither infectious virus nor complete viral DNA molecules were rescued from the treated cells. A heterogenous collection of DNA molecules containing SV40 sequences was generated following treatment with DMBA. Restriction enzyme analysis of the amplified DNA molecules in the Hirt supernatant revealed that not all sequences in the integrated SV40 inserts are present. The possibility that the amplification of SV40 sequences is a reflection of a general gene amplification phenomenon mediated by carcinogens is discussed.

Key words: gene amplification *tsA*209, DNA synthesis, benzo(a)pyrene, MNNG, DMBA, EMS, AFB_1, MCA, DBA, phenanthrene, chromosomal rearrangement, carcinogenesis, transformation, Chinese hamster, short-term assay, amplification, onion skin replication, origin of replication

Although 80% of human cancer is the result of exogenous environmental agents, very little is known about the molecular mechanism(s) leading to tumor formation. Cancer is a multistep process. Epidemiological studies of human cancer indicate that several specific heritable changes are necessary to change a cell into a malignant cell [1]. It has been widely assumed that mutation represents at least one stage in this process since the carcinogenic and mutagenic potencies of several physical and chemical agents are closely related [2,3]. However, mutagens and nonmutagenic carcinogens have been found to induce chromosomal rearrangements. Chro-

Abbreviations: BP, Benzo(a)pyrene; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; DMBA, 7,12 dimethyl benz(a)anthracene; Ph, phenanthrene; EMS, ethyl methanesulfonate; DBA, dibenz(a,c)anthracene; AFB_1, aflatoxin B_1; MCA, 3-methyl cholanthene.

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mosomal changes are associated with various types of malignancies, such as leukemias, meningiomas, and gliomas. Furthermore, somatic cells from patients suffering from cancer-prone syndromes, such as Bloom, Fanconi anemia, and Athaxia talengiectasia, show a high level of spontaneous chromosomal instability [4]. Chromosomal aberrations, such as homogenously staining regions, which presumably represent tandem copies of the same gene [5], and double minute chromosomes, which appear to be derived from homogenously staining regions [6], have been widely reported to occur in tumors, in malignant and in neoplastic cells, but are very rare in nonneoplastic cells. These findings suggest that chromosomal changes may be a step in carcinogenesis.

Chromosomal rearrangement is now a recognized phenomenon in several areas of biology; genes rearrange during evolution [7] and in certain stages of development [8,9]. Gene amplification was also observed for several genes such as dihydrofolate reductase [10], aspartate transcarbamylase [11], and metallothionein-I [12].

The understanding of the processes by which chemical carcinogens induce chromosomal changes may be of considerable importance to the understanding of cancer. The aim of this work was to study the role of chemical carcinogens in amplification of specific genomic sequences. A model experimental system was constructed, which utilizes SV40-transformed Chinese hamster cells, coupled with a highly sensitive in situ hybridization procedure for the detection of a single cell in which viral DNA synthesis has been induced [13,14]. The transformed Chinese hamster embryo cells were selected for these studies since 1) they were found to be capable of metabolizing several carcinogens into their active intermediates, and 2) they are sufficiently semipermissive for SV40 DNA replication, such that the induction process can be readily monitored without the additional complication of subsequent cell fusion with permissive monkey cells.

Induction of SV40 DNA Synthesis in SV40-Transformed Chinese Hamster Embryo Cells by Chemical Carcinogens

The cell lines used for these studies are clonal isolates derived from a colony of Chinese hamster embryo cells infected with SV40, which appeared in soft agar medium. Treatment of SV40-transformed Chinese hamster embryo cells from clones C060, C050, and C053 with DMBA resulted in the induction of SV40 DNA synthesis, as detected by the dispersed cell in situ hybridization procedure (Fig. 1) [13]. The induction of SV40 DNA synthesis by polycyclic hydrocarbons depended on the metabolic activation of the compounds since pretreatment of C050 cells with an inhibitor of polycyclic hydrocarbon metabolism (7,8-benzoflavone) nullified the phenomenon [13].

Treatment of cells (line C050), by a variety of chemical and physical agents resulted in the induction of SV40 DNA synthesis (Fig. 2). Activation-dependent carcinogens, like BP, DMBA, AFB_1, and MCA, and activation-independent carcinogens such as MNNG and EMS induced SV40 DNA synthesis in the treated cells. A similar response was obtained after irradiation by UV or γ radiation. Noncarcinogenic compounds, like Ph and DBA, were inactive. Treatment of the cells with EMS (1.0 μg/ml), DMBA (0.1 μg/ml), AFB_1 (0.1 and 0.01 μg/ml), or with γ or UV radiation, was followed by a dramatic induction of SV40 DNA synthesis that could be monitored even when 10^4 cells were assayed, indicating that a substantial number

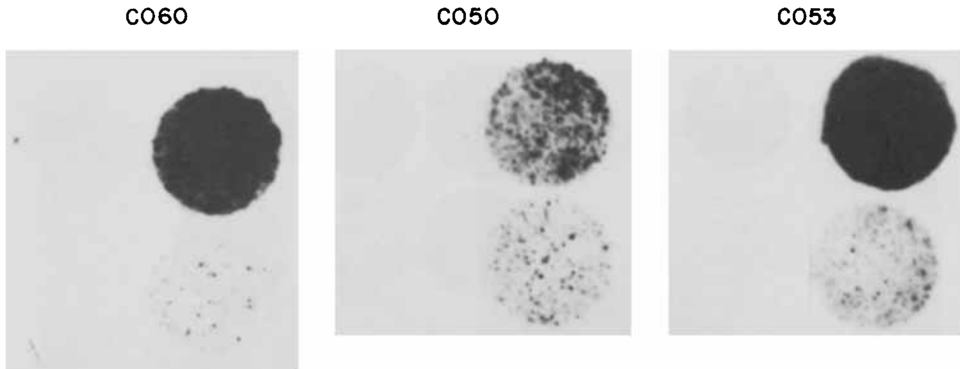

Fig. 1. Autoradiogram showing the induction of SV40 DNA synthesis in DMBA-treated, SV40-transformed Chinese hamster embryo cells from lines C060, C050, and C053. Cells from lines C060, C050, and C053 were treated with DMBA (0.1 μg/ml) for 24 h, incubated for an additional 4 days at 37°C, and then samples of 10^6 (top row) and 10^5 (bottom row) cells were trapped on nitrocellulose filters, denatured, and hybridized against (^{32}P)-SV40 DNA as described elsewhere [13]. Each panel represents the results obtained for each cell line. The right column in each panel shows an autoradiogram of control cells treated with acetone, and the left column shows DMBA-treated cells.

of the cells responded. The induction of SV40 DNA synthesis was dose-dependent, since increasing concentration to AFB_1 and MNNG increased the synthesis of SV40 DNA. Although the carcinogenic compounds were toxic to the treated cells, we found no direct relationship between cytotoxicity and inducing ability (13). A wide variety of chemicals are currently being screened in order to establish the relationship between the carcinogenic potential of these compounds and their ability to induce SV40 DNA synthesis.

The Role of the Viral "A" Gene

To elucidate the role of the viral "A" gene in the amplification of SV40 DNA sequences, we studied the effect of DMBA on Chinese hamster embryo cells, transformed by a temperature-sensitive mutant of SV40 *tsA*209 (cell line C1102) in which the transformed phenotype is under the regulation of the viral "A" gene. The transformants were treated with DMBA for 24 h and then maintained, either at 33°C (the permissive temperature) or at 40°C (the restrictive temperature). As seen in Figure 3, track A, induction of SV40 DNA synthesis occurred only when the cells were incubated at the permissive temperature. Following treatment and incubated at the restrictive temperature, no synthesis of SV40 DNA was observed (Fig. 3, track C). Similar results were obtained with three other lines of Chinese hamster embryo cells transformed by *tsA*209 [14]. In contrast, in C060 cells transformed by wild type virus, DMBA induced SV40 DNA synthesis at 37°C (the optimal temperature for wild type SV40 replication (Fig. 3D), and at 40°C (Fig. 3F). These findings demonstrate that a functional "A" gene is required for the amplification of SV40 DNA sequences.

Exposure of C1102 cells to DMBA at 40°C followed by a temperature shift to 33°C yielded high induction of SV40 DNA synthesis (Fig. 3B). C060 cells, which were treated at 40°C and then shifted to 37°C, behaved similarly ((Fig. 3E). The induction of SV4O DNA synthesis by DMBA in C060 and C1102 cells upon tempera-

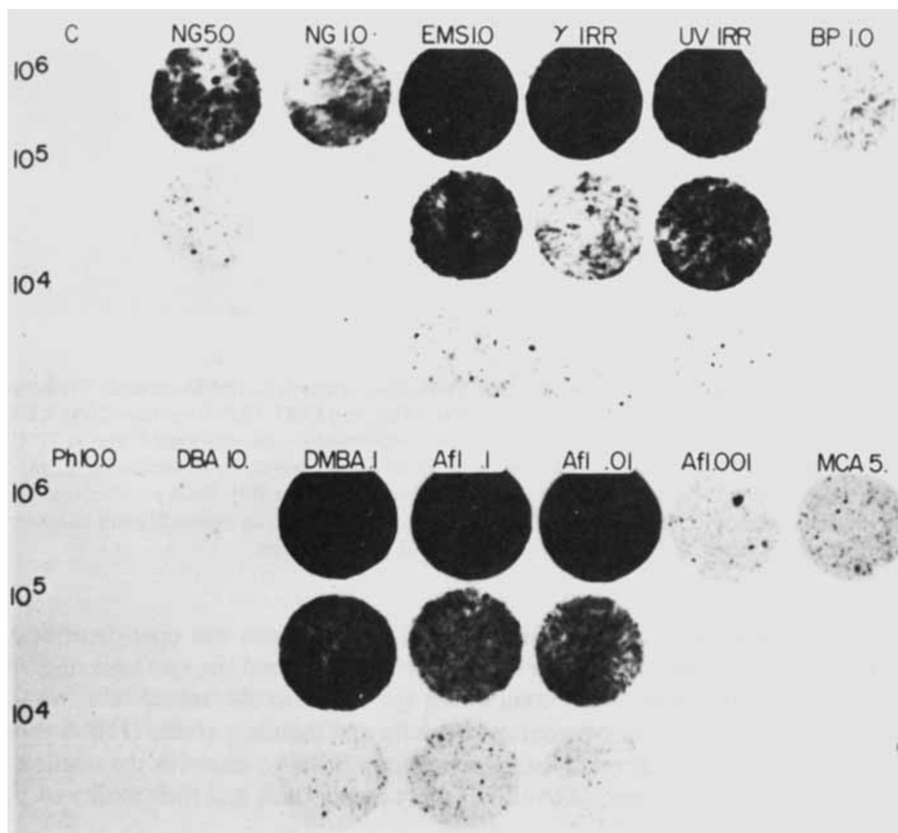

Fig. 2. Autoradiogram showing the effect of potential chemical and physical carcinogens on the induction of SV40 DNA synthesis in SV40-transformed Chinese hamster cells. C050 cells were treated for 24 h with the following compounds: 0.5% DMSO (C); MNNG (NG, 5 and 1 μg/ml); EMS (1μg/ml); BP (1.0 μg/ml); Ph (10 μg/ml); DBA (10 μg/ml); DMBA (0.1 μg/ml); AFB_1 (Afl, 0.1, 0.01, 0.001 μg/ml); MCA (5 μg/ml); γ-IRR, irradiation with γ radiation (250 r); UV-irr, UV irradiation ($505/m^2$). Samples of 10^6, 10^5, and 10^4 cells were analyzed for SV40 DNA synthesis by the dispersed cell assay, as described in Figure 1 [13].

ture shift was stronger than that obtained when these cells were treated and maintained at the permissive temperature. The increased synthesis might have resulted from the additive effects exerted by heat at DMBA. "Heat induction" was observed in untreated cells from lines C1102 and C060 upon incubation at 40°C and maintenance at the permissive temperature (Fig. 3B and E, top row), and in untreated cells from line C060, which were maintained at 40°C (Fig. 3F, top row). The temperature shift experiments indicate that l) the initial modifications mediated by chemical carcinogens are not dependent upon the viral "A"gene; 2) the expression of carcinogen-mediated modification is dependent upon the functional "A" gene, which is required for the activation of the viral origin of replication; and 3) the mechanism(s) triggered by carcinogens are also activated by the elevated temperature (heat induction).

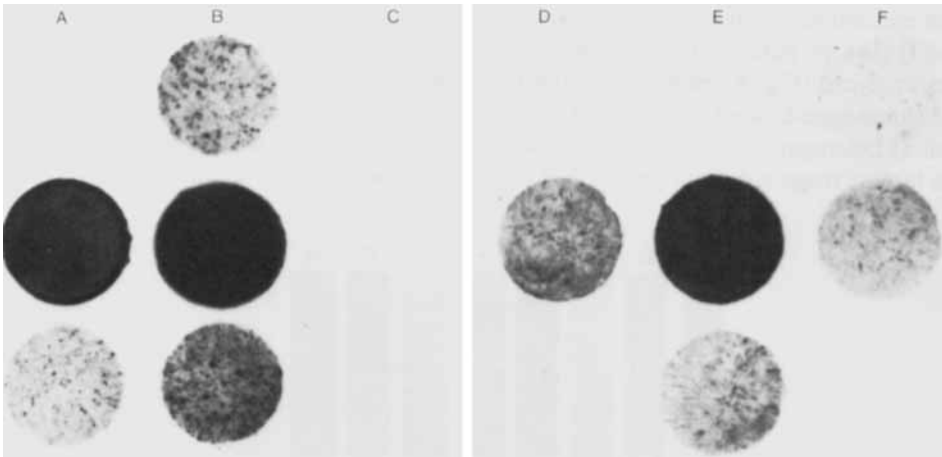

Fig. 3. Autoradiogram showing the effect of temperature on carcinogen-mediated induction of SV40 DNA synthesis in Chinese hamster cells transformed by a temperature-sensitive mutant of SV40. Cells from line C1102 (A–C) or CO60 (D–F) were treated with DMBA (0.1 μ g/ml) for 24 h, incubated for an additional 4 days at the indicated temperatures, and then assayed by the dispersed cell assay for SV40 DNA synthesis. A) C1102 cells treated and maintained at 33°C; B) C1102 cells treated for 24 h, incubated at 40°C for a further 24 h, and then shifted to 33°C for 3 days; C) C1102 cells treated and maintained at 40°C; D) C060 cells treated and maintained at 33°C; E) C060 cells treated for 24 h at 40°C and then shifted to 37°C for 4 days; F) C060 cells treated and maintained at 40°C. Top row: aliquots of 10^6 control cells treated for 24 h with 0.5% acetone. Second row: 10^6 DMBA-treated cells; Third row: 10^5 DMBA-treated cells.

Analysis of the Amplified SV40 DNA

To gain a clearer understanding of the mechanisms leading to the induction of SV4O DNA synthesis in transformed Chinese hamster cells, we studied line C060 in more detail [14]. Restriction enzyme analysis of the SV4O sequences in this cell line demonstrated 1) that all the SV4O sequences in the genome of untreated cells were associated with high molecular weight DNA, 2) that SV4O sequences are inserted in five positions in the C060 genome, and 3) that SV4O inserts within the C060 genome do not contain tandem unit-length repeats.

Following treatment of C060 cells with a variety of carcinogens neither infectious virus nor unit-length SV4O DNA sequences were detected [14]; furthermore, we were not able to detect SV4O DNA synthesis in monkey cells (BSC-1) transfected with DNA extracted from DMBA-treated C060 cells.

An analysis of SV4O DNA sequences in the Hirt supernatant of DMBA-treated cells has indicated that the carcinogen-mediated induction of SV4O DNA synthesis generated a heterogenous collection of DNA molecules that range in size from considerably larger than authentic viral DNA to smaller fragments [14]. The heterogeneity in the size of the SV4O-containing fragments can be attributed to heterogenous ends of amplified sequences, which may result from nonspecific termination sites of DNA synthesis (possibly at the site of DNA modification). Cleavage of such molecules with restriction enzymes will lead to the formation of discrete bands against a background of heterogenous fragments containing SV4O. DNA derived from the Hirt supernatant fraction of DMBA-induced cells was analyzed by restric-

tion endonuclease digestion with *Bgl* I, *Eco* RI, and *Hind* III (Fig. 4, tracks C,F and I) (14). In parallel, the high molecular weight DNA from untreated CO6O cells was analyzed (Fig. 4, tracks B, E, and H).SV4O-containing DNA molecules from the carcinogen-treated cells yielded restriction fragment classes of discrete size as well as heterogenous populations; these latter fragments were always smaller than the largest fragments containing SV4O sequences. Some of the amplified fragments

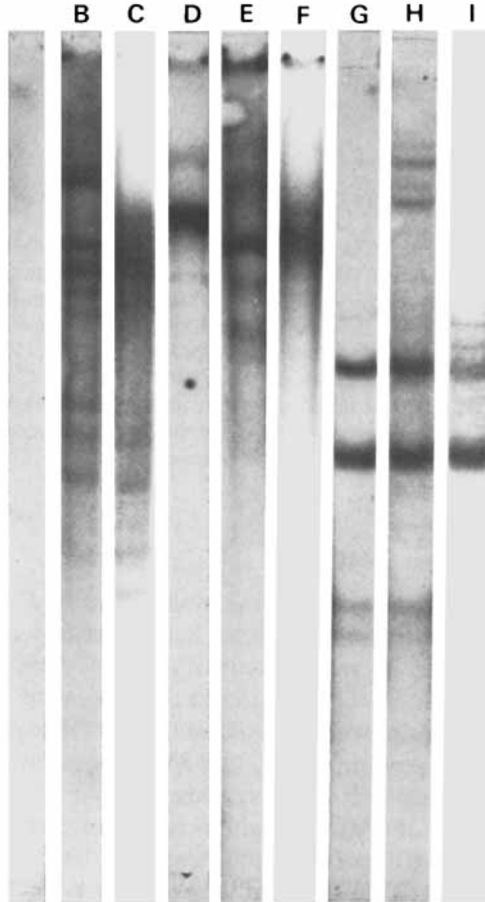

Fig. 4. Analysis of restriction endonuclease fragments derived from genomic C060 DNA and from the Hirt supernatant DNA of C060 cells treated with DMBA. High molecular weight genomic C060 DNA and DNA from Hirt supernatants of untreated or DMBA-treated cells were digested with various restriction endonucleases. The DNA fragments were electrophoresed on a 1.4% agarose slab gel and blot hybridized with (^{32}p)-SV40 DNA (14). Track A contains 1 μg of Hirt supernatant DNA from untreated cells mixed with 20 μg of normal Chinese hamster DNA. Tracks B, E, and H contain 20 μg of high molecular weight C060 DNA from untreated cells. Tracks C, F, and I contain 1 μg supernatant DNA from DMBA-treated C060 cells mixed with 20 μg of normal Chinese hamster DNA. Track D contains 50 pg of SV40 DNA markers (forms I, II, and III) mixed with 20 μg of normal Chinese hamster DNA. Track G contains 50 pg of SV40 DNA mixed with 20 μg of normal Chinese hamster DNA. DNA in tracks A–C was digested with *Bgl* I. DNA in tracks E and F was digested with *Eco* RI. Tracks G–I contain *Hind* III digested DNA. Blots containing Hirt supernatant DNA from DMBA-treated cells were exposed for 4 h whereas strips containing genomic C060 DNA were exposed for 72 h (taken from [14]).

are similar to the SV4O-containing fragments in the genome of CO6O cells. Other fragments present in the untreated parental CO6O DNA were not observed in the amplified DNA; and new fragments (which were not detected in the parental genome) appeared after induction [14]. This altered pattern of amplified DNA can be attributed to preferential amplification of specific SV4O inserts, possibly those containing viral replicons. The appearance of new SV4O-containing fragments can be ascribed to the amplification of DNA fragments that were previously undetected owing to small amounts of SV4O sequences contained in them. Alternatively, the appearance of the new bands can also be attributed to the generation of new combinations of SV4O-containing sequences as a result of duplication of existing sequences or from recombinational events during amplification. Analysis of Hirt supernatant DNA from CO6O cells treated with AFB_1 or MNNG yielded identical patterns of SV4O specific fragments.

Analysis of DNA extracted from C060 cells labeled for 1 h with ^{3}H-thymidine 48 h after benzo(a)pyrene treatment indicated that 7% of the radioactivity associated with high molecular weight DNA (Hirt pellet) was SV40-specific, whereas only 0.2% of the radioactivity associated with the low molecular weight DNA (Hirt supernatant) was SV40-specific (S. Lavi, in preparation). These findings suggest that the newly synthesized DNA molecules are associated with the chromosomal DNA. Restriction enzyme analysis of SV40-containing sequences in the high molecular weight DNA was identical to that in the low molecular weight DNA.

Analysis of the amplified DNA molecules, following cloning into prokaryotic vectors indicated that, in addition to SV40 sequences, Chinese hamster sequences are also amplified (Lavi and Gotlib, in preparation). Recently we found that specific cellular sequences are amplified in secondary cultures of Chinese hamster embryo cells following treatment with carcinogens. The biochemical and biological characteristics of these sequences are currently being investigated.

Activation of SV40 replicons by chemical carcinogens might occur by a biological mechanism similar to that occurring in prokaryotes prior to lysogenic induction, such as cleavage of a cellular repressor by proteases [15,16]. Repeated activation of replicons might lead to onion skin replication, first proposed by Sambrook et al [17]. Some of the newly synthesized DNA molecules containing SV40 sequences can be excised from the chromosome by several possible mechanisms [18] and appear in the Hirt supernatant.

Amplification of SV40 DNA sequences might reflect a more general gene amplification phenomenon mediated by chemical carcinogens. We propose that cellular replicons are activated following DNA perturbations; repeated activation of these regulatory sequences will result in an amplification of the sequences spanning the replicons and to increased expression of the genes encoded by them (S. Lavi, in preparation); alternatively, insertion of the amplified DNA sequences into new loci on the chromosome might lead to the activation of dormant genes or to the formation of new genes [19]. Such processes may lead to aberrant cellular growth and finally to the formation of cancerous cells.

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