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Carcinogen-Induced *trans* Activation of Gene Expression

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We report a new mechanism of carcinogen action by which the expression of several genes was concomitantly enhanced. This mechanism involved the altered activity of cellular factors which modulate the expression of genes under their control. The increased expression was regulated at least in part on the transcriptional level and did not require amplification of the overexpressed genes. This phenomenon was transient; it was apparent as early as 24 h after carcinogen treatment and declined a few days later.

The tumorigenic process is associated with multiple alterations of cellular structures and processes (16). Some of these changes, such as mutations, occur in the cell only as a result of a faulty mechanism (35). Other processes, such as chromosomal rearrangements (32), gene amplification (2), and changes in gene expression (39), are also utilized in normal cell regulation and differentiation (6, 33, 34, 37); however, the divergence of these processes from their natural patterns of occurrence might be related to the carcinogenic process. Carcinogens cause phenomena which accompany malignancy (1, 3, 5, 17, 19, 22, 36). We reported previously that carcinogens induce gene amplification and enhanced gene expression of simian virus 40 (SV40) and dihydrofolate reductase (17, 19, 20, 20a). Metabolic inhibitors of DNA replication induce similar effects (15, 36). Recently, we have shown that enhanced expression of two unlinked genes occurs in the same subpopulation of treated cells, suggesting the involvement of *trans*-acting cellular factors in this process (Kleinberger et al., submitted for publication). Studies on the molecular mechanisms underlying this phenomenon are presented in this paper.

Treatment of SV40-transformed Chinese hamster embryo cells (C060) (19) with a variety of carcinogens (including 7,12-dimethylbenz[a]anthracene [DMBA] and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [MNNG]) induces the amplification of SV40 sequences (19, 20a), an effect which is associated with an increase in the level of T antigen (Fig. 1A and a; 20). T-antigen synthesis was analyzed by labeling control and treated cells 2 h before their harvest, at various times after treatment. In several experiments, the specific activities of proteins from control and treated cell extracts were similar (around 10^4 cpm/μg of protein). Similar numbers of trichloroacetic acid-precipitable counts (5×10^6) were subjected to immunoprecipitation with monoclonal anti-T antibodies and to subsequent electrophoresis on polyacrylamide gels (11, 23). Carcinogen-induced enhanced expression of T antigen occurs also in the absence of SV40 DNA amplification and cellular DNA replication. Aphidicolin, an inhibitor of DNA polymerase α (13), under conditions which reduce [^{3}H]thymidine incorporation to 10 to 20% of normal values, abolished DMBA-induced SV40 amplification but did not reduce the carcinogen-mediated overexpression of T antigen (Fig. 1B and b). The amplification-independent increase in T-antigen levels was evident as early as 24 h after treatment and remained high at least up to 48 h posttreatment (Fig. 1B). The nature of the polypeptides coprecipitated with T antigen was not identified; the most prominent band migrated to the position of p53.

To study the molecular mechanisms involved in the carcinogen-mediated enhanced expression and to identify the elements controlling this process, we used the chloramphenicol acetyltransferase (CAT) assay system. By using the DEAE-dextran transfection technique (25), we introduced different eucaryotic regulatory elements linked to the bacterial CAT gene into Chinese hamster embryo (CHE) cells transformed by an origin-defective SV40 (814 cells). These regulatory elements included the SV40 early promoter and enhancer (pSV2CAT; 9), a long terminal repeat of a mouse intracisternal A particle (prcmCAT-1; 12), and the β-actin promoter (27). The plasmid containing an SV40 origin of replication (pSV2CAT) can be amplified in the T antigen expressing 814 cells after MNNG treatment (Y. Berko-Flint and S. Lavi, manuscript in preparation), while the other two plasmids, which lack viral origins of replication, are not amplified. To eliminate a possible effect of the carcinogens on DNA uptake, we applied MNNG or DMBA 4 h after the transfections. CAT assays were performed with cell lysates prepared 72 h posttreatment (at this time, under our experimental conditions, the extracts yielded maximal CAT activity). Unmodified [^{14}C]chloramphenicol and N-acetylated reaction products were separated by ascending thin-layer chromatography (9). All the regulatory elements studied facilitated enhanced CAT activity in the carcinogen-treated cells (Fig. 2A). The increased activity ranged from three- to sixfold in the absence of amplification. pSV2CAT activity reached saturation levels in this experiment; however, similar results were obtained in an independent experiment in which pSV2CAT activity in 814 cells was monitored in the linear range of the assay. A CAT construct lacking eucaryotic transcriptional elements (pSV0CAT; 9) did not express CAT activity in extracts from either control or treated cells (Fig. 2A). The amplification-independent enhanced expression of CAT constructs was not restricted to cells expressing T antigen or to transformed cell lines. Induced CAT activity was observed in carcinogen-treated secondary cultures of CHE cells transfected with pSV2CAT (Fig. 2B). A similar induction of CAT activity was observed in CHO cells stably transformed by pSV2CAT (CC24 cells; Kleinberger et al., submitted), demonstrating that enhanced CAT expression

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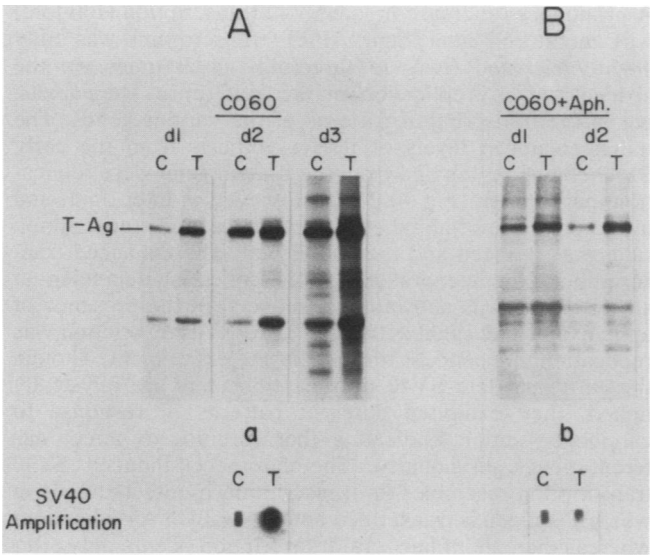

FIG. 1. T-antigen synthesis and SV40 DNA amplification in carcinogen-treated C060 cells. At 16 h before treatment, 10^6 C060 cells were seeded onto 9-cm-diameter plates. These cells were treated for 6 h with 0.5 μg of DMBA per ml which was freshly dissolved in dimethylsulfoxide. The final dimethylsulfoxide concentration in the medium did not exceed 0.5%. An equal concentration of dimethylsulfoxide without carcinogen was applied to control cells (19). (A) Cells grown without aphidicolin; (B) cells grown in medium containing 1 μg of aphidicolin per ml, which was changed every 24 h. Cells were metabolically labeled with [^{35}S]methionine (23) (50 μCi/ml, 800 Ci/mmol; Amersham Corp., England) in methionine-free medium for 2 h before harvest at days 1, 2, or 3 posttreatment (d1, d2, or d3, respectively). ^{35}S-labeled proteins (5×10^6 cpm) were immunoprecipitated (23) with monoclonal anti-T antibodies (pAb419) (11) and electrophoresed on 12.5% polyacrylamide gels. Gels were dried and fluorographed. SV40 amplification was measured by slot blot hybridization on day 3 after treatment, which was the time of maximal carcinogen-induced amplification (a and b) (17, 19). C, Control cells; T, treated cells; Aph., aphidicolin.

did not result from variations in the uptake and stability of the transfected constructs.

The induction of CAT activity by carcinogens could result from a direct interaction between the carcinogens and the overexpressed genes or could be indirectly mediated by *trans*-acting factors activated after carcinogen treatment. To prevent the direct interaction between the carcinogens and the indicator gene, we treated CHE or 814 cells with MNNG, a labile carcinogen with $t_{1/2}$ of 1.1 h (31), at various times before transfection with pSV2CAT. Figure 3 shows the enhanced CAT activity in the carcinogen-pretreated cells. Maximal enhancement in CHE cells (3.5-fold) occurred when the cells were treated 1 or 2 days before transfection. At this time, [^{14}C]chloramphenicol conversion was 3.9% in control cells and 13.6% in cells treated 2 days before transfection. The increased levels of CAT activity did not result from differences in DNA uptake, DNA stability, or plasmid amplification in the carcinogen-treated cells. Similar plasmid DNA contents were measured by dot blot hybridization in control and treated CHE cells, both shortly after transfection and at the time of harvest (data not shown). In 814 cells which facilitated pSV2CAT amplification, maximal enhancement (80-fold) was observed upon treatment with MNNG 3 days before transfection (Fig. 3). In control cells, 1% of the chloramphenicol was modified, compared with 82% modified in treated cells (Fig. 3). The differences in the temporal responses and the levels of CAT activity between the two cell systems might be attributed to the presence of T antigen and plasmid amplification in 814 cells. Thus, pretreatment with MNNG facilitated enhanced CAT expression in 814 and CHE cells and plasmid amplification in 814 cells long after the decay of the drug. These results demonstrate that a direct interaction between the carcinogen and the affected gene is not required for enhanced expression and amplification, indicating that these responses are mediated by carcinogen-induced *trans*-acting factors. Earlier studies by Nomura and Oishi (28) and by Lambert et al. (18) demonstrated that indeed viral amplification can be induced in *trans*.

To determine whether changes in transcription rates are

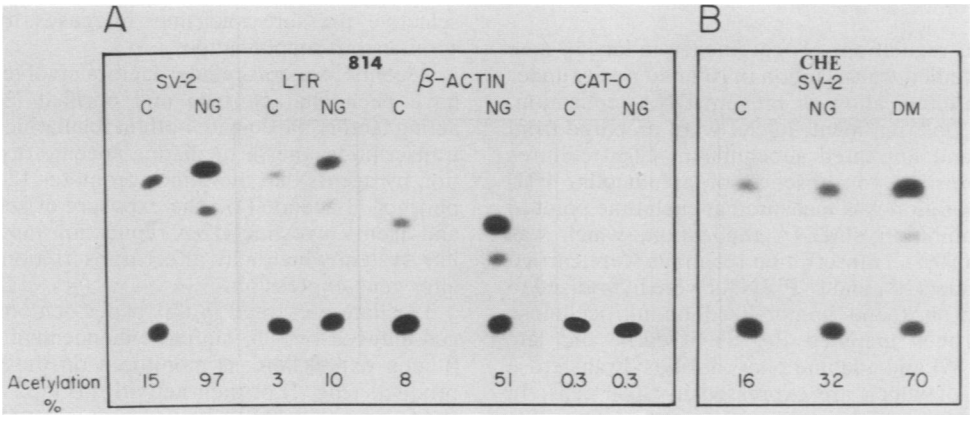

FIG. 2. Levels of CAT expression directed by different regulatory elements. Exponentially growing cells (3×10^6) were transfected in 1 ml of suspension with 1 μg of plasmid DNA by the DEAE-dextran method (25). The cells were 814 (A) and secondary cultures of CHE (B); the transfecting plasmids were pSV2CAT (SV-2) (9), prcmCAT-1 (LTR) (12), β-actin CAT (27) and pSV0CAT (CAT-0) (9). Transfected cells were seeded at 10^6 per 9-cm-diameter plate. At 4 h after transfection, 814 cells were treated for 2 h with 4 μg of MNNG (NG) per ml, and the transfected CHE cells were treated for 2 h with 2.5 μg of MNNG per ml or for 6 h with 0.5 μg of DMBA (DM) per ml, as described in the legend to Fig. 1. Cell lysates were prepared 72 h posttreatment, and CAT assays were performed (9). A total of 150 μg of protein was incubated for 60 min with [^{14}C]chloramphenicol and *N*-acetyl coenzyme A. Unmodified [^{14}C]chloramphenicol (bottom spots) and acetylated reaction products were separated by ascending thin-layer chromatography (9). Percent acetylation was determined by cutting the appropriate spots from the thin-layer chromatography plate and counting them in toluene-based scintillation fluid. C, Control.

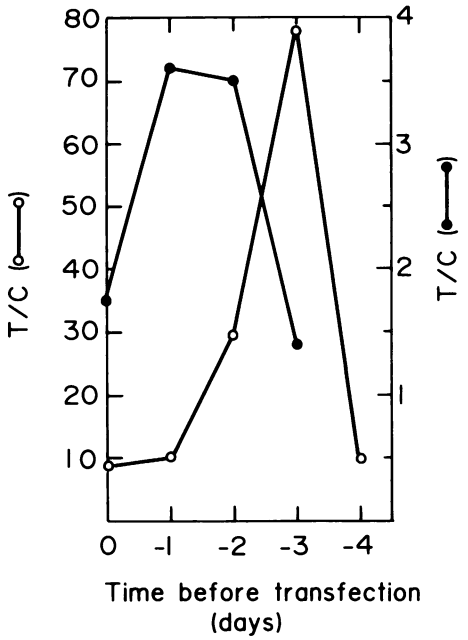

FIG. 3. pSV2CAT activity in cells treated with MNNG at various times before transfection. Exponentially growing cultures were seeded on four consecutive days (10^6 cells per 9-cm-diameter plate). At 16 h after seeding, 814 (○) and CHE (●) cells were treated for 2 h with 4 and 2.5 μg of MNNG per ml, respectively. On day 5 after the onset of the experiment, all groups of control and treated cells were harvested, and 3×10^6 cells per ml were transfected with 1 μg of pSV2CAT by the DEAE-dextran method (25). The transfected cells were seeded at 10^6 cells per 9-cm-diameter plate. One group of untreated cells was treated 4 h posttransfection (time 0). At 72 h after transfection, cell lysates were prepared, and CAT assays were performed with 150 μg of protein extracts. The CAT assays were performed in duplicates, and their time courses were determined. Only the results from the linear range of the assay are presented. The ratio of the CAT activities of treated (T) and control (C) cells is plotted as a function of the length of time between treatment and transfection. Shortly after transfection and at the time of harvest, 10^5 cells were taken from each group for the determination of plasmid DNA content (results not shown).

involved in the regulation of carcinogen-induced gene expression, we studied transcription in isolated nuclei under conditions which either allow or prevent DNA replication. At various times posttreatment, nuclei were prepared from MNNG-treated and untreated subconfluent C060 cultures grown in the presence or absence of aphidicolin. [^{3}H]thymidine incorporation was measured at each time point to determine the inhibition of DNA replication, which was found to be 80 to 90%. Transcription reactions were carried out in isolated nuclei (10), and [^{32}P]RNAs were hybridized to different DNA probes and immobilized on nitrocellulose filters. These probes included the SV40 early and late regions, β-actin (29) and adenine phosphoribosyltransferase (APRT) (21), all of which are expressed in C060 cells. In several experiments using the same numbers of nuclei ($\sim 2 \times 10^7$), 8×10^6 to 12×10^6 cpm were incorporated into RNA in reactions with control and treated cells, indicating that the overall levels of transcription did not increase in the treated cells. Figure 4 shows the increased transcription rates of all the genes studied after carcinogen treatment, under conditions which allow DNA replication. At 24 h after treatment, a dramatic increase in transcription rates was observed for the early SV40 sequences (35-fold) and for β-actin (27-fold). A pronounced increase in late SV40 transcription (100-fold) was monitored later, and APRT transcription was only slightly elevated (two- to threefold) at all times. In the absence of DNA replication, marked differences were apparent in the transcription patterns of the various genes. The initial enhanced levels of RNA synthesis from the early SV40 coding region and from the β-actin gene were replication independent (Fig. 4, 24 h). However, at later times and in the presence of aphidicolin, the increase in transcription rates was reduced and then abolished. The enhanced transcription of the late viral genes was replication dependent at all times and was substantially reduced in the presence of aphidicolin. The slight activation of APRT transcription was replication independent throughout the experiment. Though the early and late SV40 transcription units are physically linked, they exhibited different patterns of response to carcinogen action, indicating that carcinogens affect different genes dissimilarly. The carcinogen-induced SV40 transcription resembles its transcription in infected cells in which T antigen is transcribed before viral DNA replication, whereas the bulk of late viral transcription occurs only after the onset of DNA replication (38). The spectrum of genes activated after carcinogen treatment is currently being investigated.

The peak of replication-independent enhanced early SV40 transcription (Fig. 4) preceded maximal viral DNA amplification (19) (3 to 4 days posttreatment), suggesting that carcinogen-induced transcription occurs before DNA amplification. Carcinogens might activate cellular factors affecting transcription, and the enhanced transcription of these genes might lead to their amplification. However, a causal relationship between the two phenomena cannot be determined from our experiments. Alternatively, since transcription and replication share common *cis*- (7, 14, 30) and *trans*-regulatory (8) elements, activation of such components might lead simultaneously to both enhanced transcription and gene amplification. It was previously shown by Mariani and Schimke that the subpopulation of cells displaying enhanced dihydrofolate reductase expression give rise to increased numbers of methotrexate-resistant colonies containing dihydrofolate reductase amplification (24). It is possible that enhanced expression allows the cells to survive longer under selective pressure and thus increases the probability of spontaneous amplification.

Recently, several cellular factors involved in transcription have been characterized and purified (26). These *trans*-acting factors participate both in regulating the basal level of transcription and in mediating specific responses to induction by agents like the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (4). The exposure of cells to carcinogens and agents arresting DNA replication may trigger an SOS-like system which may affect transcription factors and thus alter gene expression.

The data presented in this paper demonstrates a carcinogen-induced two- to sixfold enhancement of T-antigen and β-actin expression, as monitored on the level of the gene products (Fig. 1) or their activities (Fig. 2 and 3). However, the transcription of these genes in isolated nuclei increased several ten-folds (Fig. 4). This difference might stem from the enhanced rates of transcription initiation compared with the elongation of the nascent transcripts. We have previously shown the preferential amplification of origin sequences containing the 5′ end of the SV40 transcription units (20a; Kleinberger et al., submitted). However, enhanced SV40 transcription occurs even in the absence of DNA amplification and therefore cannot be solely attributed to the

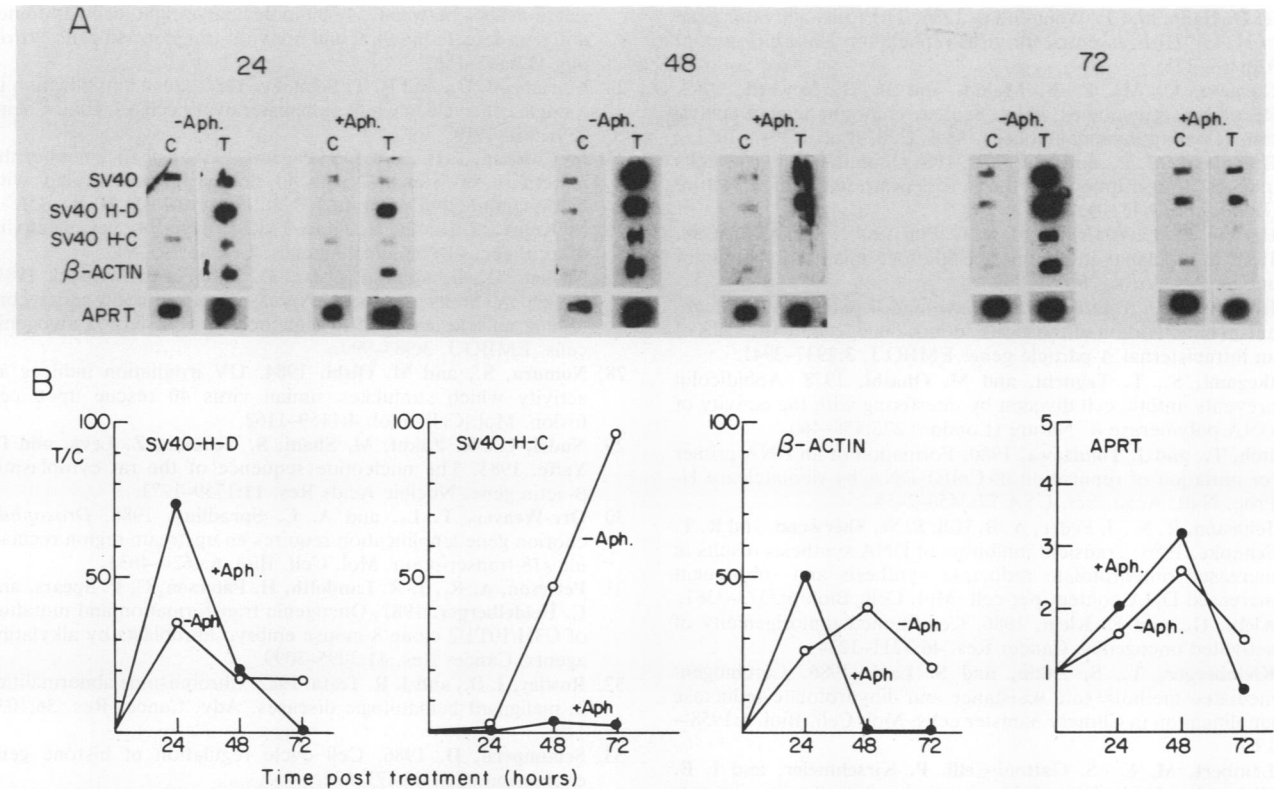

FIG. 4. Transcription in isolated nuclei from treated and untreated C060 cells. C060 cells were plated (3×10^6 cells per 15-cm-diameter dish) 18 h before a 2-h treatment with 10 μg of MNNG per ml. One group of control (C) and treated (T) cells was kept in medium containing 2 μg of aphidicolin per ml (+Aph.), which was changed every 24 h. The other cells were grown in the absence of aphidicolin (−Aph.). Nuclei were collected 24, 48, and 72 h after treatment and stored at −70°C. During days 1 and 2, the cells remained in subconfluent cultures. Transcription in isolated nuclei was carried out as described by Greenberg and Ziff (10). (A) In vitro-synthesized nuclear RNAs were hybridized to different DNA probes (5 μg of linearized plasmid DNA per slot): SV40, *Hin*dIII D fragment of SV40 (map position, 3411 to 3937) in pBR322 (SV40 H-D), *Hin*dIII C fragment of SV40 (map position, 5106 to 981) in pBR322 (SV40 H-C), pAC18.1 (β-actin) (29), and pHaprt-1 (APRT) (21). Hybridizations were carried out in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate [pH 7.2])–4× Denhardt solution (1× Denhardt solution consists of 0.02% [wt/vol] each of Ficoll, bovine serum albumin, and polyvinyl-pyrrolidone)–0.1% sodium dodecyl sulfate–0.1% tetrasodium pyrophosphate at 42°C for 48 h. The autoradiograms obtained from the hybridization reactions were subjected to computerized densitometry to quantitate the intensity of hybridization. APRT hybridization was quantitated by counting the filters in scintillation fluid. (B) The ratios of the hybridization intensities of treated and control RNAs (T/C) are plotted as a function of time posttreatment.

greater abundance of short templates containing the 5′ end of the gene. It is also possible that only a selected fraction of the transcripts is represented in the cytoplasm as stable mRNA. Aspects of this phenomenon are currently being investigated.

The novel mechanism of carcinogen action described here may be associated with the initiation of carcinogenesis. Cellular processes are controlled by a variety of regulatory molecules. The interactions between different regulators are delicately balanced to allow the normal functioning of the cell. Disturbances in the carefully maintained equilibrium, which may result from the altered expression of control elements, may lead to the escape of the cell from its normal control, thus becoming a tumor cell.

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