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Effect of Mitomycin C on Specific Gene Transcript Levels in Cultured Mammalian Cells

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The effect of mitomycin C on the accumulation of specific mRNAs was studied in asynchronously growing Swiss 3T3 cells, as well as in synchronously growing serum stimulated ts13 cells (a temperature-sensitive mutant from the BHK cell line). It was observed that the steady-state level of p53 RNA experienced some increase in 3T3 cells treated for 24 h with the drug. In addition, mitomycin when applied to serum stimulated ts13 cells increased the level of p2F1 RNA. Mitomycin diminished the level of core histone H3 RNA, a finding consistent with the inhibitory action of this compound on DNA replication.

Key words: Mitomycin C, Specific genes, Cultured cells.

Recombinant DNA technology has proved to be a powerful tool for the characterization of gene functions involved in different cell activities. Thus, some authors have reported gene sequences the expression of which appears to be specifically linked to cell proliferation, differentiation or abnormal cell functioning (2, 4, 6, 11, 14). Furthermore, it appears that such methodology may be very valuable when investigating the action, at a molecular level, of treatments of interest for biomedical purposes.

At present, a large variety of compounds have been described which, because of their cytostatic or differentiation promoting properties, may be employed as tumor therapeutic agents. Often, the action of these compounds at a cytological and biomedical level is well documented, although there is still little information on their effects on gene expression. Such is the case of mitomycin C, a DNA cross linking drug with an antineoplastic action (5, 7).

The purpose of this report is to provide with some information on the action of mitomycin C on gene expression, by measuring the steady-state levels of some specific gene transcripts in cultured mammalian cells.

Materials and Methods

Cells lines and culture conditions. — Experiments were carried out with the mouse derived Swiss 3T3 cell line, or with ts13 cells, a G_1-specific temperature sensitive mutant isolated from baby hamster kidney (BHK) cells (18). Swiss 3T3 cells were routinely grown at 37° C

in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Conditions for growth, achievement of quiescency (G_0, prereplicative blockade) and serum stimulation of ts13 cells were as earlier described (1).

Isotopic procedures. — The ability of quiescent ts13 cells to enter S phase under the appropriate stimulus was determined by serum stimulating them for different periods of time in the continuous presence of 0.5 μCi/ml of (methyl-^{3}H)-thymidine (6.7 Ci/mmol), after which the percentage of cells with labelled nuclei was estimated by autoradiography. The activity of ^{3}H-thymidine incorporation into DNA was measured by serum stimulating quiescent ts13 cells for 30 h, the labelled precursor (1 μCi/ml) being present only during the last hour of stimulation. The amount of radioactivity into trichloroacetic acid precipitable material was then determined by scintillation counting procedures (13).

Plasmids. — The clones used in this work were: human histone H3 clone pF0422 (obtained from Dr. G. Stein, Gainesville, Florida); mouse p53 clone p21-p53 (kindly provided by Dr. L. Kaczmarek, Warsow, Poland) and clone p2F1, derived from a ts13 cDNA library (6).

Isolation on Northern blot analysis of RNA. — Total cytoplasmic RNA was obtained essentially as described by MANIATIS *et al.* (12). Namely, cells were lysed for 5 min with 0.6% Nonidet P40 in an isotonic buffer (150 mM NaCl, 1.5 mM $MgCl_2$ in 10 mM Tris-HCl, pH 8.0) containing 10 mM vanadyl ribonucleosides (New England Biolabs). After centrifugation (at 1,000 *g* for 5 min) the supernatant was recovered and extracted several times with phenol-chloroform and once with chloroform-isoamyl alcohol, after which the nucleic acids were recovered by ethanol precipitation. Samples of RNA were denatured with formaldehyde, fractionated in agarose (1.1%)-formaldehyde gels (10) and blotted to nitrocellulose (19). Gel blots were hybridized with an excess of nick translated, ^{32}P-labelled plasmid probes (17).

Results and Discussion

Although G_2 blockade seems to be the main effect of mitomycin C on cell cycle progression in eukaryotic cells (8, 9), it has been described that this compound may also interfere with DNA replication (3, 8). This was confirmed by applying mitomycin, either as a pulse or as a continuous treatment, to serum stimulated ts13 cells. By autoradiographical analysis at different periods of release from the G_0 blockade, it was observed that the drug reduced the fraction of cells capable of reaching S phase (fig. 1a). In addition, those cells that were able to enter S phase in mitomycin treated populations exhibited a reduced intensitiy of labelling, especially under continuous treatment with the drug (fig. 1b). The combination of both effects, namely the number of labelled cells and the intensity of labelling may account for the considerable decrease in the rate of ^{3}H-thymidine incorporation in populations of mitomycin treated cells, as measured by quantitative radiochemical means (fig. 1c).

No attempts were made to analyze systematically other cytological parameters. However, some cell enlargement, the adoption of irregular shape and frequent micronucleation were features often observed after long term treatment with the drug.

Changes in the levels of specific gene transcripts. — To obtain preliminary information on the influence of mitomycin C on the expression of particular genes, the steady-state levels of p53 RNA and

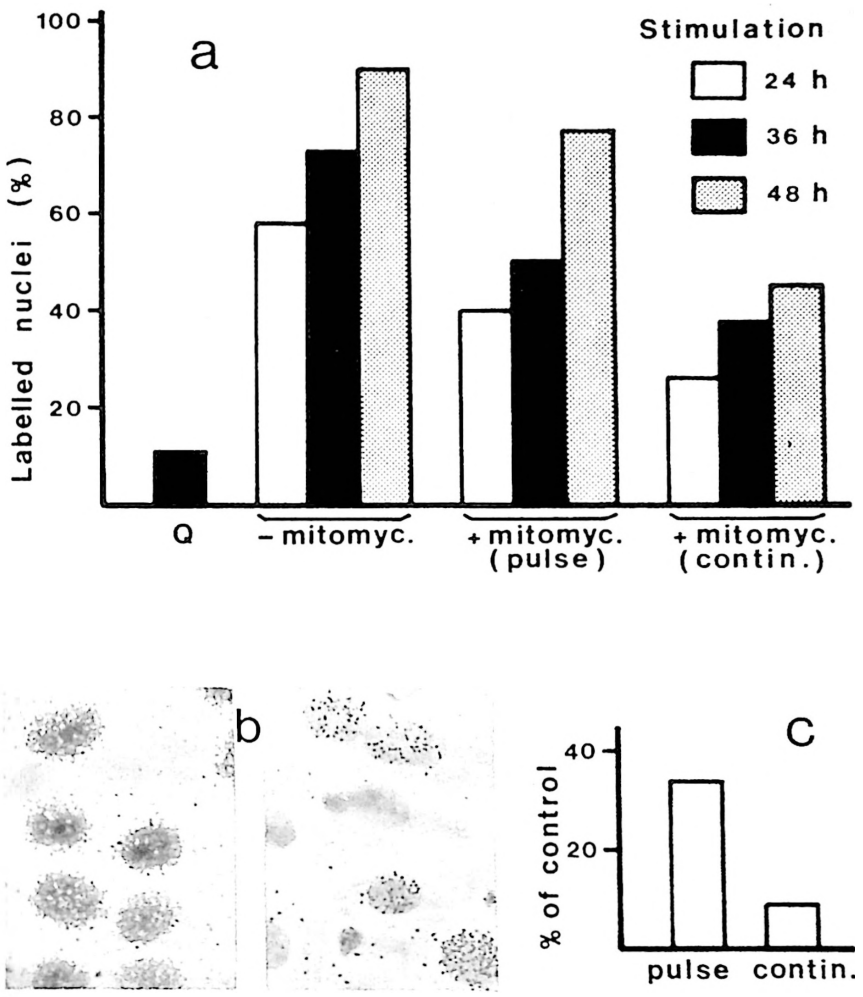

Fig. 1. *Effect of mitomycin C on ^{3}H-thymidine incorporation in serum stimulated ts13 cells.* (*a*) Quiescent ts13 cells were serum stimulated for the indicated periods of time in the continuous presence of ^{3}H-thymidine. The percentage of cells with labelled nuclei was determined by autoradiography. + *Mitomyc. (contin.)*: the drug (0.5 μg/ml) was present during the whole period of stimulation. + *Mitomyc. (pulse)*: quiescent cells were first serum stimulated for 5 h to allow chromatin to decondense, and then for 2 h more in the presence of the drug (0.5 μg/ml); afterwards they were extensively washed with Hank's balanced salts and incubated to the end of the corresponding stimulation period in the absence of the drug. *Q*: percent of labelled nuclei in a population of quiescent, drug untreated cells after 36 h labelling. (*b*) A detail showing the intensity of labelling after 36 h of serum stimulation in the absence (left) or in the presence (right) of mitomycin C. (*c*) The activity of ^{3}H-thymidine incorporation was measured in mitomycin C treated cells, and expressed as a percentage of the incorporation in control (drug untreated) cells. The results represent the mean value of two different experiments. Conditions of mitomycin administration were as in (a). For other experimental conditions, see Materials and Methods. Control value: 59,623 ± 774 c.p.m.

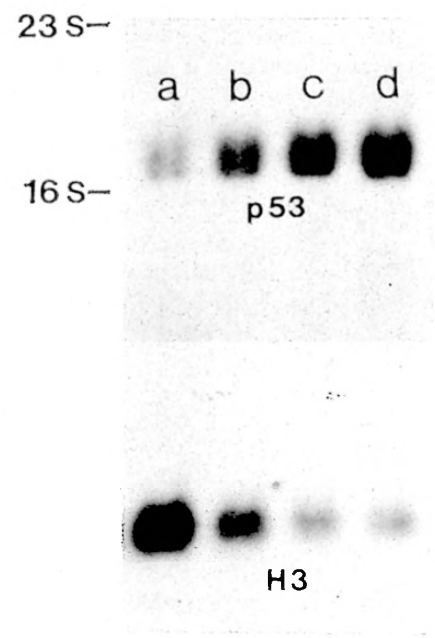

Fig. 2. *Effect of mitomycin C on specific gene transcript levels in asynchronously growing cells.* Total RNA (20 μg per lane) extracted from exponentially growing Swiss 3T3 cells untreated (a) or treated for 24 h with 0.2 (b), with 0.5 (c) or with 2.0 (d) μg/ml of mitomycin C, and allowed to recover for another 24 h, was used for Northern analysis. The same filter was successively probed with p53 and H3 clones.

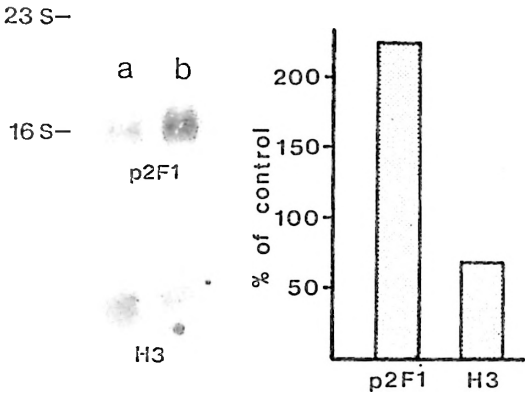

Fig. 3. *Effect of mitomycin C on specific gene transcript levels in serum stimulated cells.* Quiescent (G_0-arrested) ts13 cells were stimulated with serum for 16 h in the absence (a) or in the continuous presence (b) of 0.5 μg/ml of mitomycin C. Total cytoplasmic RNA (20 μg per lane) was used for Northern analysis. The same filter was successively probed with p2F1 and H3 clones. Values in the histogram, showing the extent of mitomycin induced changes, were obtained by densitometric readings of the Northern blots.

histone H3 RNA were measured in populations of asynchronously growing 3T3 cells by means of Northern blot analysis. p53 is the gene carrying informatioin for the transformation related p53 protein, but it is also expressed to a certain level in non-transformed proliferating cells (15). Regarding core histone H3 RNA, high level accumulation of this transcript seems to be closely associated with the ongoing of S phase (16). It was observed that a 24 h treatment with mitomycin C induced a moderate, albeit readily detectable increase in the level of p53 RNA, while H3 RNA was, on the contrary, decreased (fig. 2).

The interpretation of results obtained using asynchronously growing cells may be made difficult by the possible interference of the given treatment with different stages of cell cycle progression. This is the case of mitomycin C, which seems to interfere at least with G_2/M transition (8, 9) and S phase progression (see above). For this reason, new experiments were carried out in which the drug was applied to ts13 cells synchronously released from a G_0, prereplicative arrest. The level of p2F1 RNA —a transcript which undergoes a transient overaccumulation shortly after serum stimulation (6)— and H3 RNA were measured at 16 h of serum stimulation, a time which for most cells corresponds to mid-late G_1, although some of them (25-30%) have already reached the S phase. As indicated in fig. 3, p2F1 attained higher levels in mitomycin treated cells than in untreated cells. On the contrary, the amount of H3

RNA was slightly decreased as a consequence of the treatment.

While the decrease of H3 RNA levels in mitomycin C treated cells may be considered as a consequence of the inhibitory action of this compound on DNA synthesis, given the close association between core histone expression and DNA replication activity (16), a satisfactory explanation for the increase of other gene transcript levels cannot be offered at present. In fact, the overaccumulation of p2F1 RNA observed in synchronously growing ts13 cells does not appear to be related to the interference of mitomycin with DNA replication since (a) the increase is already observed at 16 h of stimulation, which for most cells is a pre-replicative stage, and (b) the blockade of DNA replication by hydroxyurea or cytosine arabinoside has no effect on p2F1 expression (ALLER and HIRSCHHORN, submitted). Also, any possible relation between p2F1 overexpression and the ability of mitomycin to provoke G_2 arrest must be excluded under the present experimental conditions. It seems then that the alteration in the expression of certain genes in mitomycin treated cells should be examined at the level of the direct action of this compound on the transcriptional machinery, probably as a consequence of the changes in chromatin structure caused by its DNA cross linking capacity.

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Resumen

Se estudia el efecto de la mitomicina C sobre el nivel de acumulación de ciertos mRNAs, utilizando células Swiss 3T3 en crecimiento asincrónico así como células ts13 (mutante termosensible de la línea celular BHK) estimuladas a proliferar sincrónicamente por suero. El nivel de RNA p53 experimenta un cierto incremento en células tratadas durante 24 horas con la droga. Además, la administración de mitomicina a células estimuladas a proliferar por suero incrementa el nivel de acumulación del RNA de histona H3, lo cual es congruente con la acción inhibidora de este compuesto sobre la actividad de replicación del DNA.

Palabras clave: Mitomicina C, Genes específicos de ciclo, Células en cultivo.

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