**INTRODUCTION**

The p53 gene is one of the most frequently mutated genes found in human cancer (1). Its product, the nuclear phosphoprotein p53 has been characterized for its ability to interact with DNA tumor viruses by binding the protein products of these viruses (2,3). p53 protein has been implicated in the control of the cell cycle and several reports have shown that constitutive expression of wild-type p53 can inhibit the cell growth of transformed cell lines, particularly by a block in G1 phase (4—6). p53 can bind DNA and function as a transcriptional regulator (7—9) and the DNA sequences recognized by p53 have been reported (10-12). In addition, the protein has been shown to interact with Hsp 70 and with the product of the mdm-2 gene (13). The binding to the latter blocks the ability of p53 to activate transcription (14).

In human ovarian carcinomas mutations in the p53 gene have been described in all the stages of this malignancy (15,16). In this class of tumors genetic alteration in chromosome 17, which contains the locus of the p53 gene, have been reported (17—20).

Utilizing a temperature sensitive p53 mutant (pLTRp53cGval135) which expresses mutant p53 at 37°C and a wild-type like p53 at 32°C, we transfected a human ovarian cancer cell line (SKOV3) which does not express endogenous p53. Among the different clones obtained, we selected three clones. Two were obtained from simultaneous transfection of p53 and neomycin resistance expression plasmids (SK23a and SK9), the other was obtained from transfection experiments utilizing the neomycin resistance gene only (SKN). Introduction of mutant p53 did not alter the morphology or growth characteristics of this ovarian cancer cell line. Upon shifting to the permissive temperature, a dramatic change in morphology and growth rate was observed in SK23a and SK9 cells that is associated with the presence of a wild-type like p53. SKN and SKOV3 cells maintained at 32°C did not change morphology and only slightly reduced proliferation. Both SK23a and SK9 cells did not show evidence of apoptosis when measured up to 72 hours of maintenance at 32°C. In contrast to what observed in other cell lines, SK23a and SK9 cells maintained at 32°C were not blocked in G1, but they were accumulated in G2-M. This accumulation was transient and could be due either to a blockade or to a delay in the G2 progression. No down-regulation of c-myc was observed in p53 expressing clones when shifted to the permissive temperature. In these conditions gasd45 mRNA expression was highly stimulated in SK9 and SK23a cells but not in SKN cells. In both clones Gas1 mRNA was not detected either at 37°C or 32°C. This system represents a new and useful model for studying the effect of the absence of p53 (SKOV3 or SKN), presence of mutated p53 (SK23a and SK9 kept at 37°C) or wild type p53 (SK23a and SK9 kept at 32°C) on the mechanism of response of cancer cells to DNA damaging agents.
under G418 selection. Among the different clones obtained, we selected two, SK23a and SK9 which express both neomycin resistance and murine p53 and another, SKN which expresses neomycin resistance only and was obtained from cells transfected only with pSVNeo. The clones were maintained at 37°C under selection with 500 μg/ml of G418. For the experiments, the cells were seeded without antibiotic.

**Northern and Southern analysis**

Total RNA was extracted with the guanidium isothiocyanate/cesium chloride gradient technique (22) and fractionated through 1% agarose formaldehyde gels. Genomic DNA was extracted according to standard procedure (23) and fractionated through 0.8% agarose gel after restriction enzyme digestion.

Both RNA and DNA gels were capillary transferred to a nylon membrane (Genescreen plus, Dupont) in 10×SSC. The nylon filters were baked for two hours at 80°C and hybridized at 42°C in 50% deionized formamide, 10% dextran sulphate, 0.1% SDS, 1M NaCl. The probes utilized were the 1.8 Kb XbaI fragment of the human p53, the EcoRI fragment of the human H2A histone subcloned in bluescript plasmid, the EcoRI−Clal fragment of the human c-myc gene, the GAS1 gene, the actin gene and the gadd45 gene. All the probes were 32P-labeled with the Megaprime kit (Amersham). The gadd45 c-DNA probe was obtained by RT-PCR using oligonucleotides spanning the entire coding region, derived from the published human sequence (24), and subsequent subcloning into pMOSBlue T-vector (Amersham).

**Immunofluorescence**

Cells were seeded on glass coverslips and fixed with 3% paraformaldehyde in PBS containing 2% sucrose for 15 minutes at room temperature. After three PBS washes the cells were permeabilized with 0.5% Triton X-100 in Hapes buffer (20mM Hapes, 300mM sucrose, 50mM NaCl, 3mM MgCl2) for 5 minutes at 4°C, washed twice with PBS and twice with PBS-0.1% BSA. Primary antibody PAbl22 (1:40 dilution from ascitic fluid) obtained from a hybridoma cell line was then added in PBS-0.1% BSA for 30 minutes at 37°C. Coverslips were washed in PBS and FITC antibody goat anti-mouse IgG (Sigma) was then applied (1:100 dilution) for 30 minutes at 37°C. After further washes with PBS, glass coverslips were mounted in Mowiol 4-88 (Hoechst, Germany) and observed in a Zeiss Axiophot photomicroscopy equipped for epifluorescence (Carl Zeiss, Germany). Fluorescent images were recorded on KodaK TMAX 1600 films.

**Flow cytometric analysis**

Monoparametric conventional cell cycle analysis using Propidium Iodide (PI) were performed with a FACS Star Plus instrument (Becton Dickinson, USA) coupled with a Hewlett Packard Consort 30 computer system. (25). To assess the cell cycle phase percentages, the method of Baisch et al. was used (26).

**DNA fragmentation**

DNA fragmentation was measured by gel electrophoresis in SK23a SK9 and SKN kept for different times at 32°C. Briefly, cells were detached and lysed in hypotonic buffer (5 mM Tris–HCl pH 8, 10 mM EDTA, 0.5% Triton X-100) for 20 minutes on ice. Low molecular weight DNA was separated from high molecular weight DNA by centrifugation at 4°C at 12000 rpm for 20 minutes.

Soluble DNA was treated with RNase A (100 μg/ml) for 1 hour at 37°C, followed by proteinase K (200 μg/ml) for 2 hours at 50°C. DNA was then extracted sequentially with phenol, phenol–chloroform and chloroform and precipitated with ethanol before electrophoresis on 1.5% agarose gels.

**RESULTS**

We utilized a temperature sensitive mutant of murine p53, p53val135, which can transform cells at 37°C, behaving like a mutant p53 and which, upon shifting to 32°C, assumes a wild-type like conformation of p53. With this plasmid we were able to obtain different clones of SKOV3 cells (cells that express neither wild-type nor mutant p53) expressing murine p53. Direct transformation with a plasmid encoding a wild-type p53, either human or murine, under constitutive promoter, failed in obtaining any positive clone (data not shown). Under G418 selection, we selected two clones, namely SK23a and SK9, which were obtained by transfection with both p53 expression plasmid and neomycin expression plasmid and one clone (SKN) which was obtained by transfection with neomycin expression plasmid alone.

Fig. 1 shows Northern analysis in SK23a, SK9 and SKN clones (panel B). As it can be seen only the clones SK23a and SK9 (lanes 3 and 5) express a p53 mRNA corresponding to the expected size of the murine gene while SKN (lane 4) does not show any mRNA hybridizable with p53 probe. Lanes 1 and 2 represent respectively mRNA extracted from a human (mcf-7) and murine (M5076) cell line utilized as positive controls respectively for the human and murine mRNA. This pattern of expression was maintained when the cells were shifted to the permissive temperature of 32°C (data not shown).

In panel A is reported a representative Southern blot for SK23a cells. Besides the bands normally present in the parental SKOV3 cell line which are also present in SKN cells, SK23a shows a band corresponding to the length of the gene inserted which

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**Figure 1.** Panel A: Southern blot showing the presence in SK23a genome of the 2.1 Kb (indicated with an arrow) cDNA of the murine p53 which is absent in SKN. MW are the molecular weight standards. Panel B: Northern blot analysis of p53 expression in SK23a (lane 5) SK9 (lane 3) and SKN (lane 4) kept at 37°C. Lanes 1 and 2 represent respectively the hybridization from a human (mcf-7) and murine (M5076) cell lines showing the different length of the murine and human p53.
hybridized with the human p53 probe. Also for SK9 the presence of p53 murine gene inserted into the genome was verified (data not shown).

Fig. 2 reports the immunofluorescent staining with pAb122 (recognizing both wild-type and mutated p53) in SKN, SK23a and SK9 cells. As it can be seen SK23a and SK9 cells express p53 protein which is absent in SKN cells. This immunofluorescence was evident either at 37°C or after 48 hours of maintenance at 32°C.

We have evaluated the effect of shifting the temperature from 37°C to 32°C on the morphology of the clones expressing or not expressing p53. Fig. 3 shows that both the parental cell line SKOV3 and the neomycin-transfected clone SKN did not change significantly the gross morphology from 37°C to 32°C (panels a,c versus panels b,d respectively). In contrast the SK23a and SK9 clones, when shifted for 48 hours to 32°C (panel f,h) dramatically changed their typical morphology at 37°C (panel e,g) assuming a flattened form and increasing in size. It is also evident from the figure that at 32°C the cell density was lower. This is particularly evident for the clones SK23a and SK9 in respect to SKOV3 and SKN cells.

When the two clones were tested for their ability to grow in culture, we observed that at 37°C their growth curves were...
superimposable to that of the parent line SKOV3 (Fig. 4). When the cells were seeded at 37°C and 24 hours later shifted to 32°C a block in the growth was observed with the clones SK23a and SK9 which at this temperature express a wild-type like p53. SKN and SKOV3 grew at 32°C at the same rate which is, however, much slower than the rate at 37°C. This slow down in the growth was reversible since the growth rate returned to the original level when all the clones were shifted back to 37°C (data not shown).

DNA from SK23a, SK9 and SKN cells was analyzed for DNA fragmentation after different times up to 72 hours of maintenance at 32°C. In these conditions no evidence of DNA fragmentation was observed (Fig. 5 panel B), and the clones expressing the wild-type p53 (SK23a and SK9) were not different from the mock transfected clone SKN despite the differences observed in the growth characteristics (see fig. 4). The figure shows (panel A) that also at early times after shifting to 32°C DNA fragmentation was not found (the data presented are for SK23a) in conditions in which a typical ladder indicative of apoptosis (lane M) was obtained from a murine T cell clone (CTTL) deprived for 16 hours of IL-2 and here used as positive control.

The cell cycle analysis was evaluated in SKOV3, SKN, SK23a and SK9 after different time-intervals of maintenance at 32°C (Fig. 6). In SKOV3, the cell cycle phase distribution did not change significantly shifting the temperature from 37°C to 32°C. In SKN only after 12 hours at 32°C there was a moderate increase in G2-M cell fraction not evident at 24, 36 and 48 hours. In both SK23a and SK9 cells no increase in the proportion of G1 cells was observed. SK23a cells showed an accumulation of cells in G2-M phase of the cell cycle up to 48 hours of maintenance at 32°C. Also in SK9 cells, even if in a moderate amount, the tendency to accumulate in G2-M was seen up to 48 hours.

We then analyzed the early changes in mRNA expression in SK23a, SK9 and SKN cells after shifting the temperature to 32°C. Fig. 7 (panel A) reports the expression of histone H2A, c-myc and p53 mRNA after 1, 3, 6, 12 and 24 hours of maintenance at 32°C. p53 mRNA was present only in transfected cells and did not change significantly during temperature shifting. Histone H2A and c-myc were expressed at similar level in the two lines and did not change in the first 24 hours. We checked also the expression of Gas1 mRNA, which is expressed in quiescent (Go) cells, but we were not able to detect it at any time after shifting to the permissive temperature (data not shown). Panel B reports mRNA gadd45 and actin expression in the three clones after different times of maintenance at 32°C. As it can be seen the gadd45 mRNA levels were not changed in SKN cells during the maintenance at the permissive temperature, while in SK23a clone as early as 1 hour after shifting the temperature the levels were increased and maintained 24 hours later. The same was observed in SK9 clone for which the levels at 0 and 24 hours were reported.

DISCUSSION

Mutations of the p53 gene have been found in many cancer cells (1). The wild-type gene has been reported to function as a growth suppressor gene and in many cancer cell lines the introduction of wild-type p53 resulted in growth arrest (27–30). In human ovarian cancer the presence of high percentage of mutations in the p53 gene has been reported (15). We have selected a human ovarian cancer cell line SKOV3, which was reported to have rearrangements at the genomic level for p53 resulting in no expression of this gene (16). In this cell line we failed to obtain any clone expressing the human wild-type p53 gene by transfection with a plasmid containing the coding region of the human p53 under constitutive promoter control. By using a temperature-sensitive mutant murine p53 we were able to obtain different clones expressing p53. This plasmid, pLTRp53cGvall35, described by Michalovits et al. (21) allows the introduction of mutant p53, which does not interfere with cellular growth at 37°C, but upon shifting the temperature to 32°C, expresses a wild-type like p53. Like other cell lines transfected with wild type p53, a slow down in growth rate was found in SK23a and SK9 cells. This slow down in growth was found to be reversible in experiments in which temperature, after a period at 32°C was shifted back to 37°C and both clones started to grow as the parental cell line SKOV3. The SK23a and SK9
clones kept at 32°C for 48 hours changed their morphology profoundly assuming a flattened form and increasing in size. In other reports in which cell lines of different origin were used, the arrest of the growth due to the presence of wt p53 was accompanied by a blockade of the cells in the G1 phase of the cell cycle (5,6,21) and by the appearance of apoptosis (28,31–34). In our clones the expression of wild-type p53 altered the growth characteristics. As far as the cell cycle phase distribution, the shift of temperature to 32°C induced no increase in the percentage of cells in G1, a decrease in the percentage of S phase cells and an increase in G2-M phases, more evident in SK23a than in SK9 cells. The accumulation in G2 phase was reversible and may be due either to a transient G2-M block or to a slower progression of cells through G2-M phases. Although the reports published so far using this temperature sensitive mutant plasmid indicate that p53 expression causes a block in G1, there is an indication by Kastan et al. (35) that high dose of radiation, which presumably induces high levels of p53, results in both G1 and G2 arrest. Our finding suggest the possibility that p53 induces a G2 accumulation, but further demonstrations are needed to draw a conclusion on this aspect. It has recently been shown for another tumor suppressor gene (the Rb gene) that an accumulation at G2 in cells transfected with this gene is a possible event (36). Under our conditions we did not see apoptosis as assessed by evaluating DNA fragmentation in 200bp multimers. In this human ovarian cancer cell line, under conditions where p53 was expressed, shifting the temperature to the permissive one of 32°C did not alter expression of the histone H2A and c-myc mRNAs. In other systems c-myc expression has been reported to be down regulated at early times after shifting of cancer cells transfected with p53vall35 to 32°C (31). The histone H2A was selected for observation since its distribution showing that cells are mainly accumulating in G2-M (44–47). It has been proposed that the G2 blockade is a mechanism of protection against the mutagenic and lethal DNA damage chemically or physically induced allowing DNA repair before the cell undergoes mitosis (48,49). It has been speculated that in cells expressing the mutated p53 the efficiency of the cell cycle check point mechanism may be altered, thus increasing the cell sensitivity to DNA damaging agents (50,51). The cell lines described in the present study i.e. SKOV3 and SKN not expressing p53, SK23a and SK9 at 37°C expressing p53 in a mutated form and at 32°C expressing wt p53, appear potentially useful experimental models to elucidate the role of p53 in the mechanism of action of anticancer drugs causing DNA damage.

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REFERENCES
