INTRODUCTION
A common response of all living organisms to abnormally high temperatures or other environmental or metabolic insults is the stimulation of the expression of a group of proteins termed heat-shock proteins (HSPs), which may serve to protect the cells from the lethal consequences of stress (Lindquist and Craig, 1988). The stress response is accompanied by other cellular phenomena which include the inhibition of the expression of normally synthesized proteins (Lindquist, 1986), the inhibition of DNA synthesis and cell proliferation (Roti Roti et al., 1992) and the induction of apoptosis (Sikora et al., 1993).

One of the best known morphological effects of heat-shock is the production of alterations in the cytoskeleton structure. For instance, it may cause disorganization of the network of cytoplasmic intermediate filaments, to form juxtanuclear or perinuclear structures (Falkner et al., 1981; Welch and Suhan, 1985; Collier et al., 1993); destruction of the microtubule network (Lin et al., 1982); disintegration of normal actin structures including stress fibers (Iida et al., 1986); and appearance of intranuclear actin rod-shaped bodies (Welch and Suhan, 1985; Iida et al., 1986). In addition, it has been shown that heat-shock decreases the phosphorylation degree of the nuclear lamins A and C, which are intermediate filament-like proteins (Krachmarov and Traub, 1993). By contrast, very little is known about the effects of stress treatments on the synthesis of cytoskeletal proteins.

In the present report we analyze the effect of two typical stress agents, namely heat-shock and cadmium chloride, on the expression of the major cytoskeletal proteins in U-937 cells, a human promonocytic leukemia cell line (Sundström and Nilsson, 1976). The results indicate that the stress treatments do not only alter vimentin organization, but they also cause an increase in the amount of this intermediate filament protein. Materials and methods

MATERIALS AND METHODS
Cell culture and treatments
The U-937 cells used were mycoplasma-free. The cells were grown in suspension in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO₂ atmosphere at 37°C. Cells were seeded in 100 mm plastic dishes at a concentration of 10⁶ cells/ml and maintained in continuous logarithmic growth by passage every 2-3 days.
days. Actinomycin D (Sigma Química, Madrid, Spain) was dissolved in absolute ethanol at 5 mM and applied to the culture at the desired final concentrations. For heat-shock, the cultures were transferred to an oven at 42°C. For recovery after the treatments, the cells were collected by centrifugation, washed once (in the case of heat-shock) or three times (in the case of cadmium treatment) with pre-warmed (37°C) RPMI medium, and resuspended in pre-warmed cadmium-free medium. As controls, unheated cadmium-untreated cells were subjected to the same manipulations as treated cells. Inhibition of cell proliferation and permeability to trypan blue were used as criteria to evaluate the toxicity of the treatments.

**Measurement of differentiation markers**

Nitro blue tetrazolium (NBT, Sigma Química) reduction was measured by incubating 10⁶ cells for 30 minutes at 37°C in 1 ml of PBS containing 0.1% NBT and 0.15 mM 12-D-tetradecanoylphorbol-13-acetate (TPA, Sigma Química), after which 0.2 ml of 5 N HCl was added and the cells kept for 1 hour at room temperature. Upon centrifugation, the cell pellet was resuspended in 0.5 ml dimethyl sulfoxide and the absorbance recorded by spectrophotometry at 560 nm. The surface expression of CR3 (CD11b/CD18) leukocyte integrin was determined by indirect immunofluorescence combined with flow cytometry using the Bear 1 (anti-CD11b) monoclonal antibody, as previously described (Aller et al., 1992).

**Immunoblot and immunofluorescence assays**

For immunoblot assays, cells were washed with Ca²⁺- and Mg²⁺-free phosphate buffered saline (PBS) and lysed in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol. After boiling for 2 minutes at 98°C, the protein extracts were separated on SDS-polyacrylamide (10%) slab minigels (Laemmli, 1970). Electrophoretic blotting and immunological detection of proteins were carried out essentially as described by Towbin et al. (1979). The following mAbs were used as first antibodies: a mouse anti-human HSP70 which specifically recognized the stress-inducible form (clone 9F2F34-5, Stress Gene, Biotechnologies Corp, Victoria, Canada); a mouse anti-chicken actin (Amersham International, Little Chalfont, UK); a mouse anti-chicken β-tubulin (Amersham); and a mouse anti-porcine vimentin (Amersham). As the second antibody, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Copenhagen, Denmark) was used. The filters were developed with an enhanced chemiluminescence western blotting detection kit (Amersham), following the procedure described by the manufacturer.

For immunofluorescence assays, cells were washed with PBS and collected on glass slides using a cytopsin. Upon fixation and permeabilization for 15 minutes with cold absolute methanol, the cells were firstly incubated for 1 hour at room temperature with the anti-vimentin mAb in the presence of 1% bovine serum albumin, then washed three times with PBS, and incubated again for 45 minutes at room temperature with fluorescein-conjugated sheep anti-mouse IgG (Amersham) in the presence of 1% bovine serum albumin. The cells were examined by fluorescence microscopy.

**Gel retardation assays**

Nuclear extracts from 1- to 2×10⁷ cells were prepared according to the method of Schreiber et al. (1988) and stored at −70°C. The partially complementary oligonucleotides 5'-GGCTAGTGATGAGT-CAAGCCGGATC-3' and 5'-GGGATCCGGCTGACTATCAC- TAG-3' were synthesized in a Gene Assembler Plus (Pharmacia LKB, Uppsala, Sweden) and used to prepare a double-strand oligonucleotide containing an AP-1-recognizing sequence (wild type AP-1). The probe was labeled with [γ-³²P]dCTP (3,000 Ci/mm, New England Nuclear, Boston, MA, USA) following the method of Sambrook et al. (1989). The oligonucleotides 5'-GGCTAGTGATGCTGAAAGCCGGATC-3' and 5'-GGGATCCGGCTGACGAT- CACTAG-3' were also synthesized and used to prepare an AP-1 mutant oligoprobe. The binding reaction was carried out for 15 minutes at room temperature in 20 μl of binding buffer (25 mM NaCl, 60 mM KCl, 2 mM 1,4-dithiothreitol, 2% Ficoll, 0.1% (v/v) Nonidet P-40, 0.1 mg/ml bovine serum albumin, 25 mM Hepes, pH 7.4) containing 5 ng of the labeled probe, 8 μg of total nuclear proteins, 2 μg of poly(dC/dC), and 2 μg of salmon sperm DNA. To examine the specificity of binding, the reaction was carried out in the presence of excess wild-type or mutated AP-1 unlabelled probes, and in the presence of 1 μl of a polyclonal anti-jun antibody or pre-immune serum (obtained from Dr R. Bravo, Bristol-Myers Squibb, Princeton, NJ, USA). The samples were electrophoresed in 4% polyacrylamide gels, and the gels dried and autoradiographed.

**RESULTS**

**Cell growth, cell differentiation and HSP70 expression**

We firstly investigated the capacity of pulse treatments with heat (2 hours at 42°C) or cadmium chloride (3 hours at concentrations ranging from 25 to 150 μM) to affect the subsequent proliferation activity of U-937 cells, since proliferation inhibition is a criterion of stress (Roti Roti et al., 1992). The treatments transiently decreased proliferation, which was gradually reinitiated at hour 36 of recovery in the case of heat-shock and 100 μM cadmium, and at hour 60 in the case of 150 μM cadmium (Fig. 1A). The treatments caused a slight increase in the number of non viable cells, as measured by trypan blue penetration at hour 12 of recovery. In addition heat-shock and cadmium caused a transient increase in the expression of differentiation markers, namely the surface accumulation of the CD11b/CD18 leukocyte integrin (Hickstein et al., 1989) and the capacity to reduce NBT (Collins et al., 1980) (Fig. 1B). This suggests that stress treatments induce the functional differentiation of myeloid cells, as earlier observed by Richards et al. (1988) using HL-60 human promyelocytic cells. As a criterion of stress at the molecular level, the amount of the stress-responsive protein HSP70 was measured at different times of recovery by immunoblot assays. Both heat-shock (Fig. 2) and 100 μM cadmium chloride (result not shown) produced a transient increase in HSP70 which was already detected at the end of the treatment (hour 0 of recovery), reached the maximum at hours 12 to 24, and decreased thereafter to control
levels. However, the increase in HSP70 produced by 150 μM cadmium persisted even at 120 hours of recovery (Fig. 2).

Expression of cytoskeletal proteins
To analyze possible effects of the stress treatments on the expression of cytoskeletal proteins, the accumulation levels of actin, β-tubulin and the intermediate filament protein vimentin were measured at different times of recovery by immunoblot assays. It was found that the levels of both actin and tubulin, which were already elevated in non-stressed cells, remained unaltered during recovery from heat-shock (Fig. 3) and cadmium treatments (results not shown). By contrast the level of vimentin, which was low in non-stressed cells, underwent a significant increase during recovery from treatments. In the case of heat-shock (Fig. 3) and 100 μM cadmium chloride (result not shown), the increase reached the maximum at hour 24 of recovery and decreased thereafter to control levels. However, in the case of 150 μM cadmium the amount of vimentin remained elevated even at 120 hours of recovery (Fig. 3).
The changes in vimentin content were corroborated by fluorescence microscopy. The frequency of cells with detectable amounts of cytoskeleton-associated vimentin was increased at hour 24 of recovery from heat-shock (Fig. 4B) in relation to untreated cells (Fig. 4A), to decrease again at hour 72 (Fig. 4C). However, a considerable fraction of cells was still positive for vimentin at hour 72 of recovery from treatment with 150 µM cadmium (Fig. 4D).

The fluorescence microscopy assays also revealed changes in vimentin organization. The well-organized vimentin network present in unstressed cells (Fig. 4E) disappeared 3 hours after heat-shock (Fig. 4F) or 12 hours after cadmium treatment (Fig. 4G), having the appearance of diffuse fluorescence. The network was partially formed again at 24 hours of recovery after heat-shock (Fig. 4H).

**Vimentin mRNA accumulation**

It is known that HSPs may interact with and stabilize other proteins, including cytoskeletal components (Nishida et al., 1986; Lee et al., 1993). Since the kinetics of accumulation of HSP70 (Fig. 2) and vimentin (Fig. 3) during recovery from stress treatments overlapped, we asked whether the increased expression of the intermediate filament during stress could be exclusively regulated at the protein level. To investigate this question, the vimentin mRNA content was measured by northern blot assays. Some of the obtained results are shown in Fig. 5. It was found that the amount of vimentin mRNA was increased during recovery from heat-shock and cadmium treatments, indicating that the stress agents also stimulated vimentin expression at the RNA level.

**c-jun and c-fos expression, and AP-1 binding activity**

Earlier works indicated that stress agents stimulated c-jun and
Heat- and cadmium-induced vimentin expression as well as AP-1 binding activity in some cell types (Bukh et al., 1990; Jin and Ringerertz, 1990; Sikora et al., 1993). In addition, we previously observed that the increase in vimentin expression caused by differentiation inducers in U-937 cells seemed to be mediated by the stimulation of AP-1 binding (Pérez et al., 1994). Hence, we found it of interest to measure the binding of this transcription factor in stressed U-937 cells. It was found that 150 μM cadmium chloride transiently stimulated AP-1 binding at 2 to 5 hours of recovery, to decrease thereafter (Fig. 6). The treatment also stimulated the expression of c-jun and c-fos protooncogenes, which encode proteins constitutive of AP-1 (Fig. 7). Similar results were obtained using 100 μM cadmium chloride (results not shown). By contrast, heat-shock failed to significantly stimulate both AP-1 binding activity (Fig. 6) as well as c-jun and c-fos expression (result not shown).

**Vimentin mRNA stability**

It has been reported that stress treatments caused stabilization of specific gene transcripts (Andrews et al., 1987; Bukh et al., 1990; Edington and Hightower, 1990). For this reason, we wanted to determine whether the stress treatments could affect the vimentin mRNA stability. With this aim, actinomycin D was applied for increased time periods to untreated cells, as well as to cells allowed to recover for 24 hours after heat-shock and cadmium treatments. Incubations longer than 5 hours were omitted, since actinomycin D caused significant cell damage. The results in Fig. 8 indicate the rate of vimentin mRNA decay.
was slower in stressed cells than in untreated cells (Fig. 8). Under the assayed conditions (24 hours recovery from stress), transcript stabilization was considerably higher in cadmium-treated cells than in heated cells.

DISCUSSION

The results in this work indicate that heat-shock and cadmium chloride do not only cause the disorganization of the vimentin network, but also induce an increase in the amount of this intermediate filament protein in U-937 human promonocytic cells. The treatments do not alter the accumulation of the other major cytoskeletal components, namely actin and tubulin. Earlier results by Courgeon et al. (1993) indicated that stress agents increased the synthesis of actin, tubulin, and a 46 kDa intermediate filament protein in Drosophila cells. In addition, Wachsberger and Coss (1993) showed that heat-shock increased the amount of nuclear C lamin in CHO hamster fibroblasts. In both cases, the overaccumulation of intermediate filaments was only demonstrated at the protein level. Our present results indicate that the stress-elicited vimentin increase in U-937 cells is regulated at least in part at the RNA level, excluding the possibility that it could be exclusively a consequence of protein stabilization.

Stimulation of vimentin expression has been commonly observed upon mitogen activation of quiescent fibroblasts (Ferrari et al., 1986), as well as during the differentiation induction of myeloid leukemia cells (Rius and Aller, 1992). In both cases, vimentin stimulation seemed to be the result of increased transcription mediated by the AP-1 responsive elements present in the promoter of this gene (Rittling et al., 1989; Rius and Aller, 1992; Perez et al., 1994). This is not the case in stressed U-937 cells, even though the stress treatments induced the expression of differentiation markers. In fact, heat-shock increased the vimentin mRNA level without significantly affecting AP-1 binding activity (a result which also contrasts with the reported effect of heat in other cell systems: Sikora et al., 1993). Cadmium chloride stimulated AP-1 binding, but there was a clear uncoupling between the time of AP-1 stimulation (from 2 to 5 hours of recovery) and the time of maximum increase in vimentin mRNA (from 24 hours of recovery onwards). The present observations suggest that the increase in vimentin mRNA content in stressed U-937 cells is the result, at least in part, of transcript stabilization. The stabilized RNA appears to be a mature, normally sized vimentin transcript (2.0 kb in size) capable of translation. Stress-mediated stabilization has also been observed in the case of other gene transcripts, such as c-myc mRNA in Hyon pre-B lymphocytes (Bukh, 1990), c-fos mRNA in HeLa cells (Andrews et al., 1987), and HSP70 and HSP23 mRNAs in Drosophila cells (Peterson and Linquist, 1989) and chicken cells (Edington and Hightower, 1990), respectively. Nevertheless, it is probable that other mechanisms operating at the translational or transcriptional level may also contribute to the increase in vimentin expression. Actually, the human vimentin gene promoter possesses regulatory elements other than AP-1 which might modulate transcription in either a positive or negative fashion (Rittling and Baserga, 1987; Lilienbaum and Paulin, 1993).

The functional significance of vimentin stimulation by heat-shock and cadmium chloride is unknown. Since the stress treatments induced the expression of differentiation markers, a possibility is that vimentin might form part of, and even regulate, the stress-induced differentiation process, as it was previously demonstrated using other inducers (Bernal and Chen, 1982; Rius and Aller, 1992). Nevertheless, it must be noted that the mechanism responsible for vimentin increase in cells treated with typical differentiation inducers is not the same as that operating in stressed cells, as commented above. An alternative explanation is that vimentin may be involved in the acquisition of stress tolerance, a function commonly attributed to HSPs (Li et al., 1982). In fact, our experiments showed that the expression of vimentin partially coincided with that of HSP70 in stressed U-937 cells. In addition, Lee et al. (1992) observed that Chinese hamster mutant cell lines with increased levels of vimentin exhibited increased heat-resistance in relation to the wild-type cells, although the mutant cells did not exhibit alterations in HSPs levels. Hence, it is possible that the overaccumulation of vimentin or other intermediate filaments is one of the factors which contribute to make the cells resistant to environmental stresses.

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REFERENCES


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