P3HR-1 and virus isolated from Daudi cells have the entire U2 domain deleted (*9, 19*) and do not express an EBNA2 protein (*15*) (Fig. 3). The absence of U2 from these growth-transformed cells is in apparent conflict with the proposed role of EBNA2 in growth transformation. However, after virus-induced growth transformation, the function of EBNA2 may have been superseded by the increased expression of c-*myc* which is known to be activated in these Burkitt tumor cell lines (*23, 24*). In support of the hypothesis that deletion of U2 occurred long after growth transformation and c-*myc* translocation is the observation that Jijoye, the Burkitt tumor cell line from which P3HR-1 is derived, has a normal-sized U2 domain (*9*).

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Rapid Switching of Plant Gene Expression Induced by Fungal Elicitor

Abstract. *The pattern of messenger RNA synthesis in suspension-cultured bean cells (*Phaseolus vulgaris *L.) was analyzed by blot hybridization and in vitro translation of newly synthesized messenger RNA. The RNA was separated from pre-existing RNA by organomercurial affinity chromatography after in vivo labeling with 4-thiouridine. The elicitor induced the synthesis of messenger RNA's encoding phenylalanine ammonia-lyase, chalcone synthase, and chalcone isomerase, three enzymes of phenylpropanoid metabolism involved in the synthesis of isoflavonoid-derived phytoalexins. This is part of a rapid and extensive change in the pattern of messenger RNA synthesis directing production of a set of proteins associated with expression of disease resistance.*

The natural process of disease resistance in plants involves inducible defense mechanisms such as the accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyproline-rich glycoproteins and proteinase inhibitors, and increases in the activity of certain hydrolytic enzymes (*1*). Such responses can be induced not only by infection but also by glycan, glycoprotein, or lipid elicitor preparations obtained from fungal and bacterial cell walls and culture filtrates and in some cases by structurally unrelated artificial elicitors or mechanical damage (*1, 2*).

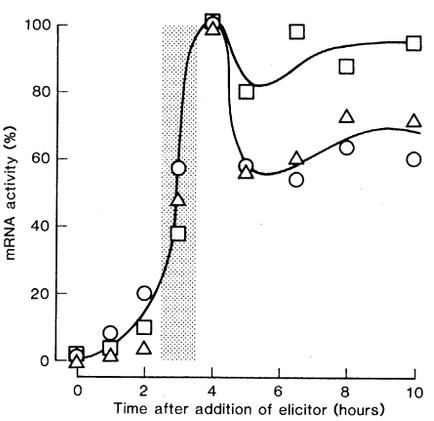

Fig. 1. Labeling of bean cells with 4-thiouridine during elicitor induction of mRNA activities encoding PAL (○), CHS (△), and CHI (□). Cells (*18*), were labeled for 1 hour (shaded area) with 4-thiouridine (1 millimolar; Sigma) and [5,6-^{3}H]uridine (0.04 micromolar, 38.4 Ci/mmol; New England Nuclear). For measurement of mRNA activities, total RNA (isolated by extraction directly into phenol) (*19*) was translated in vitro with a rabbit reticulocyte lysate translation system. Labeled enzyme subunits were visualized by fluorography following separation by immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*6*). The mRNA activity was defined as the incorporation of [^{35}S]methionine into immunoprecipitable subunits as a percentage of incorporation into total protein, and was expressed relative to the activity at maximal induction (4 hours).

Biological stress causes extensive characteristic changes in the pattern of protein synthesis that are related to expression of specific defense responses (*3–6*). Induction of messenger RNA's (mRNA's) encoding phytoalexin biosynthetic enzymes and hydroxyproline-rich glycoproteins after stress has been observed by means of RNA blot hybridization with appropriate cloned DNA sequences as probes (*7, 8*). These observations suggest that specific changes in gene expression might be an early stage in plant defense. To test this hypothesis we have studied the effect of elicitor on the pattern of RNA synthesis in suspension-cultured bean cells (*Phaseolus vulgaris* L.) by means of a technique based on in vivo labeling with 4-thiouridine (*9*).

Treatment of bean cells with a fungal elicitor prepared from *Colletotrichum lindemuthianum* causes rapid coordinated increases in the rate of synthesis of three enzymes of phenylpropanoid biosynthesis, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI), concomitant with the onset of accumulation of phaseollin and related phenylpropanoid-derived isoflavonoid phytoalexins (*5*). The increase in enzyme synthesis reflects increases in the corresponding mRNA activities which, together with modulation of the apparent stability of the biosynthetic enzymes in vivo, are responsible for the marked increases in enzyme activity that regulate expression of the defense response (*5, 6*). Using cloned CHS complementary DNA (cDNA) sequences as probes in RNA blot hybridizations, a close correspondence between induction of hybridizable mRNA and increased mRNA activity is observed (*8*).

Maximum rates of increase in the mRNA activities encoding PAL, CHS, and CHI (Fig. 1) were observed between 2.5 and 3.5 hours after treatment with an elicitor prepared from *C. lindemuthianum* (*10*), which causes anthracnose

disease in beans. We have isolated the RNA synthesized in this 1-hour period (2 percent of a generation time) and equivalent RNA synthesized in 1 hour in untreated control cells by organomercurial affinity chromatography (*11*) after in vivo labeling with 4-thiouridine and [^{3}H]uridine (Fig. 2). The [^{3}H]uridine was included as a marker for uptake and incorporation into RNA of exogenous uridine analogs. Uptake of [^{3}H]uridine was not inhibited by 4-thiouridine and 40 percent of the labeled material was taken up within 1 hour. Elicitor did not affect uridine uptake and incorporation. The 4-thiouridine did not interfere with elicitor induction, but itself showed weak activity in the absence of fungal elicitor (<10 percent of the activity of the elicitor after a 3-hour exposure, <1 percent activity during the 1-hour pulse). RNA which incorporated 4-thiouridine during the 1-hour labeling period was separated from unlabeled RNA by the formation of covalent sulfhydryl bonds with the immobilized phenylmercury agarose support and, following washing, was eluted by addition of 10 m*M* 2-mercaptoethanol (*9*).

Two cycles of organomercurial affinity chromatography efficiently separated newly synthesized and preexisting RNA. The thiouridine-labeled RNA fraction (containing more than 70 percent of the incorporated radioactivity) represented 3 to 4 percent of the total cellular RNA. The specific radioactivity in this RNA fraction was at least 80-fold greater than in the corresponding unbound RNA fraction (Fig. 2C) and further cycles did not significantly change the distribution of radioactivity. Thus this procedure generated an RNA fraction which was on the average enriched 15-fold for RNA synthesized after the addition of 4-thiouridine (Fig. 2C). Specific induced messages could be enriched as much as 30-fold in this subpopulation compared to the total RNA population (Fig. 2A).

The relative abundance of CHS mRNA in the fraction containing newly synthesized RNA from elicitor-treated cells was about 100-fold greater than in the equivalent fraction (also labeled for 1 hour) from untreated control cells (Fig. 2A). This suggests that increases in CHS mRNA are due to an increased rate of synthesis of CHS mRNA. An alternative hypothesis, that elicitor inhibited CHS mRNA turnover, would require the half-life of CHS mRNA to be shorter than the 1-hour labeling period. In fact, unlabeled mRNA represented a considerable proportion of the total CHS mRNA population in control cells at the end of the labeling period, which suggests that this RNA does not have a short half-life in untreated cells. Furthermore, the ratio of newly synthesized to preexisting CHS mRNA was greater in elicitor-treated cells (3.5 to 1) than in control cells (0.9 to 1). These observations indicate that elicitor caused a marked increase in the rate of CHS mRNA synthesis and that this was the major factor contributing to increased CHS mRNA levels in elicitor-treated cells (*12*).

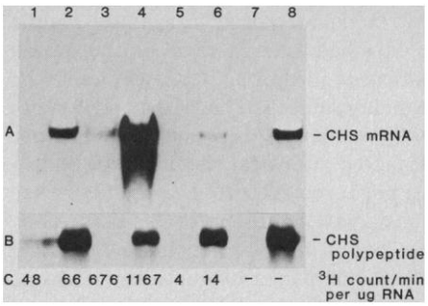

Fig. 2. Induction of CHS mRNA synthesis in elicitor-treated bean cells. CHS mRNA synthesis was monitored by RNA blot hybridization with ^{32}P-labeled CHS cDNA (*8*) (A) and in vitro translation (B) of total cellular RNA and RNA fractionated by organomercurial affinity chromatography. Total RNA was fractionated into newly synthesized (4-thiouridine–containing) RNA and preexisting RNA by two passes over Affigel 501 (Bio-Rad) columns (*11*). Purification of newly synthesized mRNA (C) was monitored by the specific radioactivity of [^{3}H]RNA (counts per minute per microgram of RNA). For measurement of CHS mRNA, 12 μg of total RNA's (lanes 1, 2, 7, and 8) and 6 μg of affinity-separated RNA's (lanes 3, 4, 5, and 6) were fractionated by agarose gel electrophoresis prior to blot hybridization. For measurement of CHS mRNA activity, 16 μg of each RNA sample was translated in vitro, immunoprecipitated with CHS antiserum, and separated by gel electrophoresis (legend to Fig. 1). Lanes 1 and 2, total cellular RNA; lanes 3 and 4, newly synthesized RNA; lanes 5 and 6, unlabeled, preexisting RNA; lanes 7 and 8, total cellular RNA from equivalent cells. Lanes 1, 3, 5, and 7 contained RNA isolated from unelicited control cells; lanes 2, 4, 6, and 8 contained RNA isolated from cells 3.5 hours after elicitor treatment.

High molecular weight (putative precursor) forms of CHS mRNA were not observed, even following prolonged autoradiographic exposure of blot hybridizations (Fig. 2A). Accumulation of precursor forms, especially in newly synthesized RNA from control cells, would be predicted if stimulation of CHS mRNA synthesis by the elicitor involved modulation of the processing of the primary transcript against a background of continuing, rapid transcription. Thus, elicitor induction of mRNA synthesis appears to have involved stimulation of CHS gene transcription.

We next compared amounts of CHS mRNA (Fig. 2A) with CHS mRNA translational activities (Fig. 2B) by a procedure involving in vitro translation of affinity-purified, thiouridine-labeled RNA, and subsequent immunoprecipitation with specific antiserum to CHS. Newly synthesized thiouridine-containing CHS mRNA (Fig. 2A, lane 4) was translated in vitro into complete, immunoprecipitable enzyme subunits with less than 5 percent of the efficiency of unlabeled CHS mRNA (Fig. 2B, lane 8). The incorporation of [^{35}S]methionine into trichloroacetic acid–precipitable material after translation from thiouridine-containing RNA was 44 percent of that obtained with the same amount of unlabeled RNA, suggesting that 4-thiouridine incorporation tends to increase the proportion of small incomplete products relative to full-length polypeptides generated during in vitro translation. Although incorporation of thiouridine reduced the efficiency of translation, there was a close correspondence between the relative increases in hybridizable CHS mRNA and translatable CHS mRNA activity in newly synthesized RNA from elicitor-treated cells compared to control cells (Fig. 2, A and B, lanes 3 and 4). Thus, comparison of the translational activities of affinity-purified, thiouridine-containing RNA from elicitor-treated and control cells is a valid approach for monitoring the effect of elicitor on the synthesis of specific mRNA species (*13*).

The translational activities of mRNA's encoding PAL and CHI, for which hybridization probes are now unavailable, were similarly determined with antisera specific for PAL and CHI. The mRNA's encoding these enzymes were present at considerably higher levels of activity in the newly synthesized RNA fraction from elicitor-treated cells than in the equivalent fraction from control cells indicating that elicitor likewise stimulated the synthesis of PAL and CHI mRNA's (*14*).

Comparison of total translation products of newly synthesized RNA from control and elicitor-induced cells (Fig. 3) shows that synthesis of some mRNA species was unaffected by elicitor. However, the synthesis of several mRNA species was decreased following elicitor treatment and elicitor markedly stimulated the synthesis of a large number of mRNA species (Fig. 3, A and B). In some cases up-regulation involved stimulation of synthesis from significant basal rates, but in many instances there was induction of mRNA synthesis from a very low or zero basal rate in control cells. Comparison of the polypeptides encoded by newly synthesized RNA (Fig. 3, A and B) and total cellular RNA (Fig. 3, C and D) indicated

that modulation of RNA synthesis by the elicitor had a major effect on the overall pattern of mRNA activity and thereby on protein synthesis. The extent and rapidity of these changes were analogous to those observed after heat shock (*15*) although, as might be expected from functional considerations, the mRNA's induced by the two stimuli are distinctly different (*4*). A number of characteristic changes in the pattern of mRNA activity associated with expression of hypersensitive resistance in hypocotyl tissue infected with *C. lindemuthianum* (*4*) were also observed in elicitor-treated cells.

Labeling with 4-thiouridine in vivo and organomercurial affinity chromatography have been used to enrich an RNA population isolated from a uridine auxotroph of *Saccharomyces cerevisiae* (*9*) for developmentally regulated mRNA's. The present study has shown that labeling with 4-thiouridine can be applied to prototrophic plant cells and that the ability to separate newly synthesized RNA from preexisting RNA can be used to study the role of RNA synthesis in cellular regulation. With our procedures, it is possible both to delineate the effect of a stimulus on the overall pattern of mRNA synthesis and to determine the effect of changes in the rate of synthesis of specific mRNA species on the synthesis and accumulation of the corresponding proteins in relation to expression of biological function. Such information cannot be readily obtained from alternative approaches such as analysis of transcripts completed in vitro in isolated nuclei (*16*).

We conclude that elicitor induces the de novo synthesis of mRNA's encoding CHS, PAL, and CHI as part of an extensive shift in the pattern of mRNA synthesis. Demonstration that specific changes in gene expression occur at an early stage in the plant defense mechanism may lead to elucidation of signal-response coupling mechanisms in plant-pathogen interactions and the development of strategies to manipulate and enhance the response of plant cells to biological stress (*17*). The technique of enrichment of defense-related mRNA's by 4-thiouridine labeling and organomercurial affinity chromatography should be useful in the identification and isolation of genes involved in plant disease resistance.

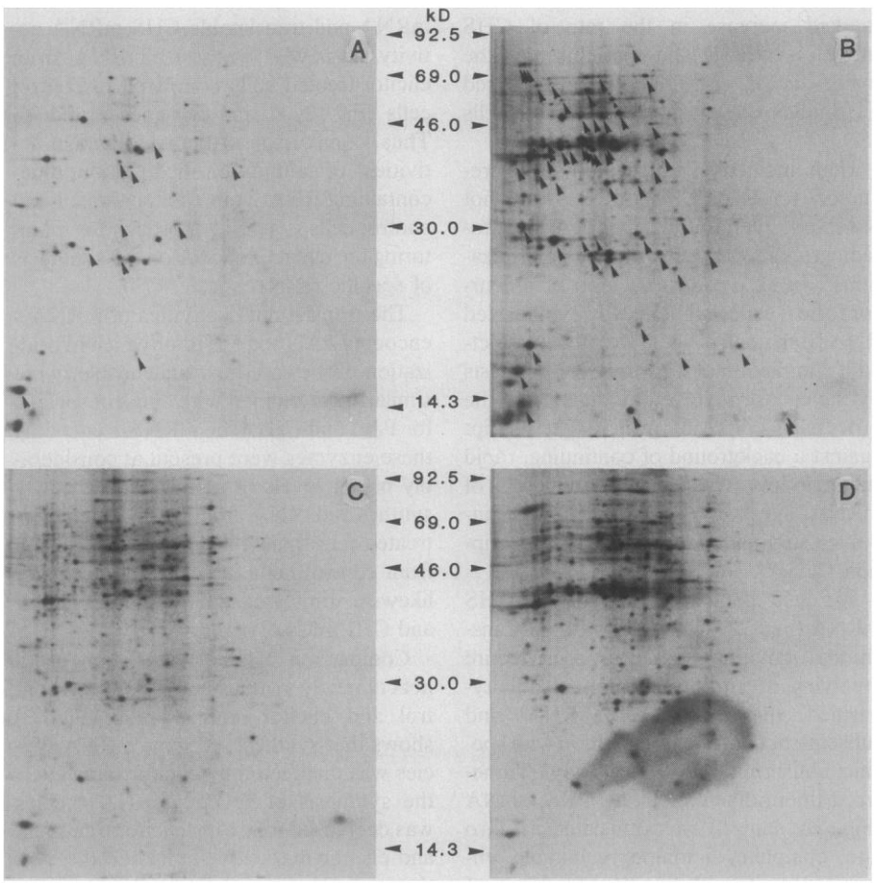

Fig. 3. Effect of elicitor on the pattern of mRNA synthesis. In vitro translation products encoded by (A) newly synthesized RNA from unelicited control cells; (B) newly synthesized RNA from elicitor-treated cells; (C) total cellular RNA from control cells; (D) total cellular RNA from elicitor-treated cells. In vitro translation products (legend to Fig. 1) from 1.6 μg of each RNA population were fractionated by the method of Garrels (*20*) with a *p*H range of 3.5 to 10 (LKB) for isoelectric-focusing (first dimension) followed by sodium dodecyl sulfate–12.5 percent polyacrylamide gel electrophoresis and fluorography. The total radioactivity of samples displayed in panels A and C was 82 percent of that in panels B and D respectively. Arrows in panel A indicate proteins encoded by mRNA's whose rates of synthesis have decreased after elicitor treatment. The arrows in panel B indicate proteins encoded by mRNA's showing increased synthesis after elicitor treatment.

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10. Elicitor, heat-released from cell walls of the pathogenic fungus *Colletotrichum lindemuthianum* [J. A. Bailey and B. J. Deverall, *Physiol. Plant Pathol.* **1**, 435 (1971)] was added to a final concentration of 60 μg of glucose equivalents per milliliter.
11. For organomercurial affinity chromatography, 5 mg of RNA in a buffer containing 0.1 percent sodium dodecyl sulfate, 0.15*M* NaCl, 50 m*M* NaOAc, *p*H 5.5, was heated to 65°C for 10 minutes. The solution was diluted with buffer (final volume, 8 ml), added to a 4-ml bed-volume of Affigel 501, and rotated for 1 hour at 4°C. The slurry was transferred to an Econocolumn (0.7 by 15 cm, Bio-Rad) and washed at room temperature with 32 ml of buffer (which eluted preexisting RNA) and then with 16 ml of buffer containing 10 m*M* 2-mercaptoethanol (which eluted thiouridine-containing newly synthesized RNA). RNA was recovered by ethanol precipitation and quantitated by absorbance at 260 nm.
12. The interpretation of 4-thiouridine–labeling experiments is based on the theoretical treatment developed for analysis of enzyme synthesis and turnover by the analogous approach of in vivo labeling with ^2H or other stable isotope, followed by resolution of labeled and unlabeled species by centrifugation to equilibrium in a density gradient [C. J. Lamb and P. H. Rubery, *Biochim. Biophys. Acta* **421**, 308 (1976)].
13. The finding of essentially complete separation of thiouridine–labeled RNA from unlabeled RNA by organomercurial affinity chromatography (Fig. 2), and the distinct patterns of polypeptide products generated by in vitro translation of affinity-purified, 4-thiouridine–labeled RNA compared with total cellular RNA (Fig. 3) suggest that labeled RNA is translationally active in

vitro and that this RNA and not contaminating unlabeled RNA is responsible for the translation products obtained. Similarly, while 4-thiouridine labeling partially inhibits reverse transcription of isolated RNA, a series of cDNA's specifically enriched for newly synthesized RNA sequences are generated using affinity-purified thiouridine-containing RNA as substrate (*9*).

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Platelet-Mediated Cholesterol Accumulation in Cultured Aortic Smooth Muscle Cells

Abstract. *Cholesterol accumulates within smooth muscle cells and macrophages in atherosclerotic lesions, thereby contributing to the progressive enlargement of these lesions. The mechanism of this cellular accumulation of cholesterol is not known. The possibility that platelets may have a role in the cellular cholesterol accumulation that occurs during atherogenesis was investigated. Incubation of thrombin-activated washed rat platelets (or platelet-free supernatants prepared from thrombin-activated platelets) with cultured rat aortic smooth muscle cells induced cholesteryl ester lipid droplet accumulation within the smooth muscle cells. No cholesteryl ester lipid droplets accumulated when smooth muscle cells were incubated with unactivated platelets. Smooth muscle cell lipid droplet accumulation occurred in the absence of serum lipoproteins and was not inhibited by mevinolin, a drug that blocks cholesterol synthesis. These findings suggest that activated platelets may release cholesterol, which can be accumulated by cells and stored as lipid droplets.*

Atherosclerosis is characterized by focal thickening of the intima of large- and medium-sized arteries. Thickening is caused in part by the accumulation of cholesterol (*1*) and cells [smooth muscle cells (*2, 3*) and macrophages (*4*)] within the intima. Much of the cholesterol accumulates within cells. Progressive thickening of atherosclerotic lesions may result in myocardial infarction or stroke. Because the mechanism of vascular cholesterol accumulation is not known, a study was undertaken to examine a possible basis for cholesterol deposition.

Although much of the cholesterol in atherosclerotic lesions accumulates within cells, some accumulates in the extracellular space (*5, 6*); this was shown with the use of filipin, a fluorescent dye that specifically binds to cholesterol. Cholesterol sometimes also accumulates at the surface of lesions (*6*), apparently within small thrombi. The association of filipin-stained cholesterol with thrombi suggested that platelets may have a role in the origin of vessel cholesterol deposits.

A study was undertaken to investigate whether platelets take part in cholesterol deposition and specifically to determine whether platelets could induce cholesterol accumulation within cultured vascular smooth muscle cells. This possibility was examined by incubating washed rat platelets with cultured smooth muscle cells of rat aorta in the absence of serum. Washed rat platelets were prepared from citrated (3.2 percent) blood removed—with a 19-gauge needle—from the abdominal vein of ether-anesthetized 3-month-old male rats (Sprague-Dawley). Platelet-rich plasma was prepared by centrifugation (3 minutes, 1500*g*, 22°C) of well-mixed blood. The platelet-rich plasma, which was prepared from the blood of each rat (~5 ml), was placed in a 15-ml conical polypropylene tube and centrifuged (30 minutes, 1400*g*, 22°C). The supernatant was removed, and platelet pellets were suspended in 10 ml of Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) containing 1 percent glucose. The platelet suspensions were centrifuged again (20 minutes, 900*g*, 22°C) and suspended in 10 ml of Ca^{2+}- and Mg^{2+}-free DPBS plus glucose. The platelets were counted with an electronic cell counter and centrifuged a final time (20 minutes, 900*g*, 22°C). The washed platelets were suspended in Ca^{2+}- and Mg^{2+}-free DPBS plus glucose at a platelet concentration of 15×10^8 per milliliter of medium. All washed platelet suspensions were pooled and held at room temperature for 2 hours before use.

Cultured rat (Wistar-Kyoto) aortic smooth muscle cells were maintained in polystyrene tissue culture flasks in minimal essential medium (MEM) containing 10 percent fetal calf serum (FCS). Confluent cultures showed the hill-and-valley pattern of growth typical of cultured smooth muscle cells (*7*).

Smooth muscle cell cultures for experiments were initiated by using trypsin to release cells from a confluent culture. Cells were plated at 160,000 cells in 4 ml of MEM and 10 percent FCS in single-well slide chambers and incubated overnight (37°C; 95 percent air, 5 percent CO_2). The medium was then replaced with 4 ml of MEM without FCS. Cell cultures remained in this medium for at least 3 days to eliminate any cholesteryl ester lipid droplets that may have accumulated during cell growth in FCS (*8, 9*). Cultures seeded at 160,000 cells were confluent at the beginning of experiments. In some experiments, cells were plated at a lower density (40,000 per chamber) to produce nonconfluent cultures.

For experiments, the desired amount of washed platelet suspension (typically 2 ml of platelet suspension containing 30×10^8 platelets) was placed in a 12 by 75 mm polypropylene round-bottom tube and centrifuged (3 minutes, 1000*g*, room temperature). The platelet pellet was then suspended in 0.5 ml of Ca^{2+}- and Mg^{2+}-free DPBS plus glucose. Smooth muscle cultures were prepared for experiments as follows. Culture medium (3.5 ml) was removed and replaced with 3.0 ml of fresh MEM containing 0.35 percent bovine serum albumin (free of fatty acid and globulin). Experimental cultures then received 16 U of human thrombin to activate platelets; control cultures received no thrombin. The concentrated washed platelets (0.5 ml) were added to designated chambers and mixed with the culture medium by repetitive pipetting (three times). Additional control cultures received thrombin without added platelets. Cultures were incubated for specified times, rinsed three times with 4 ml of DPBS, and fixed for 1 hour (room temperature) with 4 ml of 10 percent phosphate-buffered Formalin.

Cell cultures were stained specifically for cholesteryl ester to determine whether cholesteryl ester lipid droplet accumulation had occurred in the cultured smooth muscle cells. Cultures were rinsed three times with 4 ml of DPBS (5 minutes for each rinse). Unesterified cholesterol was extracted from cells with a 5-minute incubation with 70 percent