Malignant cells are known to have elevated rates of mevalonate synthesis because of increased levels and catalytic efficiency of 3-hydroxy-3-methylglutaryl-CoA reductase. Whether this increased mevalonate synthesis occurs as a consequence of increased requirements for a mevalonate-derived metabolite in response to rapid growth or whether mevalonate promotes the growth of tumor cells is unknown. To address this question, we administered mevalonate via miniosmotic pumps to nude mice inoculated with MDA-MB-435 human cancer cells. After 13 weeks of growth, tumors in mevalonate-treated mice were significantly larger than tumors in saline-treated, control mice (1.52 ± 0.26 g versus 0.81 ± 0.27 g respectively, p < 0.05). The cancer cells treated in culture with mevalonate also demonstrated increased proliferation rates associated with accelerated entry of cells into S phase. These cells had enhanced total and cyclin A-immunoprecipitable cyclin-dependent kinase-2 (CDK-2) activity, increased activating phosphorylation of CDK-2, and decreased inhibitory binding of CDK-2 to p21<sup>cip1</sup>. Our findings demonstrate that mevalonate promotes tumor growth and suggest that an increase in mevalonate synthesis in extrahepatic tissues following cholesterol-lowering therapy may explain the elevated risk of cancer shown in some studies.

HMG-CoA<sup>3</sup> reductase is the rate-limiting enzyme in cholesterol biosynthesis that catalyzes the formation of mevalonate (1). In addition to being a precursor of cholesterol, mevalonate is required for a number of cellular processes including DNA synthesis and proliferation (2). Mevalonate is also the precursor of non-sterol isoprenoids that have a variety of functions including prenylation of growth-regulating proteins and oncoproteins (3, 4). Elevated mevalonate synthesis has been reported in malignant breast (5), lung (6), leukemia and lymphoma (7), and hepatoma cells (8). Whether increased mevalonate synthesis occurs in malignant cells simply as a consequence of increased requirements for a mevalonate-derived metabolite in response to rapid growth or whether mevalonate promotes the growth of tumor cells is unknown.

HMG-CoA reductase is regulated through a multivalent feedback mechanism that is controlled, in part, by intracellular cholesterol levels (1). Cells meet their cholesterol requirements by de novo synthesis or by uptake from plasma of cholesterol-rich low density lipoprotein. A fall in circulating levels of low density lipoprotein causes a decrease in its uptake, and the resultant drop in intracellular cholesterol levels leads to a compensatory stimulation of mevalonate synthesis through up-regulation of HMG-CoA reductase (1). We have shown that a diet rich in cholesterol that raised circulating cholesterol levels also significantly decreased mammary gland HMG-CoA reductase activity (5) and inhibited the development of chemically induced mammary tumors in rats (5, 9). We have also shown in mice that a diet rich in cholesterol protects against the development of chemically induced preneoplastic lesions of the colon (10). Conversely, oral administration of the bile acid-binding resin cholestyramine to rats during the promotion phase of mammary carcinogenesis has been shown to lower circulating cholesterol levels (11), increase cholesterol synthesis in the mammary gland (12), and increase the incidence of malignant mammary tumors (11). The action of circulating low density lipoprotein cholesterol as a feedback regulator of HMG-CoA reductase has been suggested to explain these observations (5, 9–12). In support of this notion, treatment of mice with cholestyramine has recently been shown to increase prenylation of the growth-regulating protein K-Ras in the lung (13). Because statins, like cholestyramine, reduce circulating low density lipoprotein, we reasoned that the increased risk of breast, gastrointestinal, and total cancer associated with the use of statins in some studies (14–17) may be related to an increase in mevalonate synthesis in extrahepatic cells.

Statins are the class of cholesterol-lowering drugs most widely used for the prevention of cardiovascular disease. They function to reduce serum cholesterol levels by competitively inhibiting HMG-CoA reductase activity in the liver (18, 19). Recent epidemiological studies have suggested there may be an increased risk of some types of cancer following the use of pravastatin (16, 17), although not all studies have found this association (20, 21). No increased risk, however, has been associated with the use of simvastatin (22) or lovastatin (23). The mechanism whereby some statins may increase cancer risk is unknown (16). Bile acid-binding resins are used less commonly in cholesterol reduction therapy than statins but are still prescribed in North America (24). As discussed above, data from rodent studies strongly suggest that increased mevalonate synthesis mediates the growth-promoting effects of cholestyra-
The cross-sectional tumor area was calculated as described by Rose et al. with phosphate-buffered saline, and growth was continued for 60 h in accordance with the recommendations of the Canadian Council on Animal Care and the Guide to the manufacturer's instructions by quantifying the incorporation of bromodeoxyuridine into DNA using a cell proliferation enzyme-labeling procedure has been described in detail elsewhere (27). We used (Steris Isomedix, Whitby, Ontario, Canada) (28). The tumor cell inoculum size was provided by Dr. J. E. Price (Department of Cell Biology, M. D. Anderson Cancer Center, Houston, TX) (27). Cells were routinely cultured in 75-cm² flasks at 37 °C with 5% CO₂, Iscove's modified Dulbecco's medium with 1% penicillin/streptomycin (Sigma), and 5% fetal bovine serum (Invitrogen).

Malignant progression of xenografts into nude mice. To this end, 40 female nude mice (CD1-Nu/Nu) were purchased from Charles River Laboratories (Wilmington, MA) at 7 weeks of age and acclimatized for 1 week prior to study commencement. Mice were housed in microisolator cages with sterile bedding and were handled in a unidirectional laminar airflow hood. They were maintained throughout the study on a standard AIN-93G based rodent diet (Dyets, Bethlehem, PA) that was sterilized by exposure to 60Co (Steris Isomedix, Whitby, Ontario, Canada) (28). The tumor cell inoculation procedure has been described in detail elsewhere (27). We used the MDA-MB-435 human cell line, which is highly tumorigenic when xenografted into nude mice (27). Briefly, cells were grown to 70% confluence, and the medium was changed 24 h prior to harvesting by centrifugation for 10 min at 12,000 × g, and then 2–75 µg of total cellular protein was solubilized in Laemmli buffer, heated for 5 min at 95 °C, electrophoresed in SDS-12% polyacrylamide gels as described above and probed with antibodies for p21<sup>CIP1</sup>, p27<sup>kip1</sup>, cyclin-dependent kinase-2 (CDK-2), CDK-4, cyclin D1, cyclin E, or cyclin A (Upstate Biotechnology, Lake Placid, NY) and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA), and signals were detected by enhanced chemiluminescence (Amersham Biosciences). Expression patterns of p-actin and Coomassie Blue staining of total protein were used to ensure equal loading.

For immunoprecipitation, 500 µg of protein from cell lysates was diluted in lysis buffer to 1 mg/ml. Samples were preclarified by gentle rocking for 1 h at 4 °C with 30 µl of protein-agarose A beads (Sigma) followed by immunoprecipitation with anti-CDK-2 antibody (4 µg) overnight. Immunocomplexes were captured by rocking with 100 µl of protein-agarose A beads at 4 °C for 2 h. Washed immunoprecipitates were resuspended in 100 µl of 2× Laemmli buffer with 2-mercaptoethanol and boiled. Aliquots of supernatant were subjected to Western blotting using SDS-12% polyacrylamide gels as described above and probed with antibodies for p21<sup>CIP1</sup> and p27<sup>kip1</sup>.

**Histone H1 Kinase Assay**—Immunoprecipitates were prepared as described above, but in this step we used 4 µg of antibodies to CDK-2, cyclin A, or cyclin E and assayed for histone H1 kinase activity, essentially as described by Naderi and Blomhoff (30). Immunocomplexes were washed twice with lysis buffer and then three times with kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 1 mM dithioreitol, 0.1 mM NaF, 0.1 mM NaVO₄, 10 mM β-glycerophosphate). Immunoprecipitates were then incubated for 30 min at 37 °C with 25 µl of kinase assay buffer containing 25 µl cold ATP, 5 µg of histone H1 (Upstate Biotechnology), and 10 µCi of [γ-³²P]ATP (PerkinElmer Life Sciences). Reactions were stopped by adding 30 µl of Laemmli buffer with 2-mercaptoethanol followed immediately by boiling for 5 min. The products were then electrophoresed on an SDS-15% polyacrylamide gel. Radioactive bands corresponding to histone H1 were visualized and quantified using a Packard Instinct Imager (Canberra Packard Canada, Mississauga, Ontario, Canada).

**RESULTS**

**Mevalonate Promotes the Growth of Tumors in Nude Mice**—The primary purpose of this study was to determine the effect of elevated mevalonate levels on the in vivo growth of tumors derived from human cancer cells. To this end, 40 female nude mice were inoculated with MDA-MB-435 cells and then randomized and implanted with miniosmotic pumps that provided a continuous supply of either mevalonate or saline (control). Thirteen mice in each group developed growing, palpable tumors, and only these mice were included in tumor measurements (28). Accelerated tumor growth in the mevalonate-treated animals was measurable after 7 weeks (Fig. 1). The increasing divergence in tumor size resulted in significantly larger average tumor cross-sectional areas at 13 weeks in mevalonate-treated animals compared with saline-treated controls (215.0 ± 24.8 mm² versus 133.1 ± 28.1 mm², p < 0.05) (Fig. 1). Tumors in the mevalonate-treated group also weighed more at necropsy than tumors in the saline control group (1.52 ± 0.26 g versus 0.81 ± 0.27 g, p < 0.05).

Mevalonate Promotes Proliferation In Vitro by Accelerating Entry of Cells into S Phase—We determined the effect of me-
Mevalonate treatment on cell proliferation by assaying the incorporation of bromodeoxyuridine into the nuclei of replicating cells. As shown in Fig. 2A, mevalonate added to the culture medium for 60 h increased the rate of MDA-MB-435 cell proliferation. This effect was significant at 1 mM mevalonate, a concentration that elicited a 57% ± 14% increase compared with control. Treatment of cells with 5 mM mevalonate did not produce a further increase in growth.

Analysis of DNA histograms following fluorescence-activated cell sorter analysis showed that mevalonate treatment at all concentrations significantly decreased the proportion of cells in G0/G1 phase compared with untreated controls (Fig. 2B). This effect was largely accounted for by an approximate doubling of the proportion of cells in S phase (Fig. 2B). Mevalonate treatment caused no significant changes in the proportion of cells in G2/M (Fig. 2B).

**Mevalonate Alters the Expression of G1 Regulatory Proteins**—To determine whether alterations in cell cycle distribution detected by fluorescence-activated cell sorter analysis were associated with changes in expression of G1 regulatory proteins, we performed Western blot analyses of extracts of cells grown in the presence of increasing concentrations of mevalonate (Fig. 3). Cyclin E expression was similar in all groups, but expression of cyclin A increased by 20–25% in mevalonate-treated cells compared with controls. Total CDK-2 expression (active 33 kDa phosphorylated form plus inactive 34 kDa unphosphorylated form) did not change with mevalonate treatment. When the active and inactive forms of CDK-2 were resolved on a 15% gel, however, 20–25% greater levels of the activated form were present in extracts from cells treated with mevalonate. The expression of p21Cip1 decreased by ∼35% in cells treated with 0.5 mM mevalonate and by ∼55% in cells treated with 1 or 5 mM mevalonate. Mevalonate treatment also decreased p27Kip1 expression by ∼50% at all concentrations examined compared with controls.

**Mevalonate Inhibits p21Cip1 Binding to CDK-2**—Rao et al. (31) have reported that relative binding of CDK inhibitors (CDKIs) to CDK-2 may be more important than expression levels in determining their net inhibitory effect on CDK enzyme activity. To determine whether decreases in expression of CDKIs in mevalonate-treated cells are paralleled by changes in binding to CDK-2, we immunoprecipitated CDK-2 from cellular extracts and performed Western blot analyses of associated p21Cip1 and p27Kip1 proteins. Quantification by density scanning of band intensities indicated a decline of 25–35% in the relative levels of p21Cip1 complexed to immunoprecipitated CDK-2 (Fig. 3). We were unable to visualize and, therefore, to quantify a band for p27Kip1 associated with CDK-2. This was likely because of both low expression levels of p27Kip1 and low binding of this CDKI to CDK-2.

**Mevalonate Promotes Histone H1 Kinase Activity**—The histone H1 kinase activities of immunoprecipitates of CDK-2 and cyclins A and E were assayed to determine whether mevalonate increased CDK-2 activity and whether this was the result of differences in activation of the enzyme by one or both of its regulatory cyclin subunits (Fig. 4). Visualization of radiolabeled histone H1 bands showed clearly that mevalonate treatment increased CDK-2 kinase activity (by ∼3-fold at 1 and 5 mM mevalonate). This increase was found to be caused by ∼5- and 10-fold increases in cyclin A-immunoprecipitable kinase activity in cells treated with 1 and 5 mM mevalonate, respectively. Cyclin E-immunoprecipitable kinase activity increased modestly (∼1.5-fold) with 1 mM mevalonate treatment but did not change in cells treated with mevalonate at any other concentration.

**DISCUSSION**

In this study we have demonstrated that mevalonate administered to nude mice via miniosmotic pumps promotes the growth of tumors derived from MDA-MB-435 human cancer cells. This cell line was derived from a pleural effusion in a
patient with metastatic ductal adenocarcinoma of the breast (32). Until recently, this cell line was widely accepted as having originated from the breast tumor. More recent evidence, however, suggests that the cell line may have been derived from an occult melanoma (32, 33). Despite being of uncertain origin, the MDA-MB-435 cell line is a highly malignant model of human cancer. To the best of our knowledge, the present study is the first to demonstrate that mevalonate directly promotes tumor growth in vivo. In the saline-treated control group, tumors grew in accordance with growth kinetics previously reported for this cell line in nude mice (27). In the mevalonate-treated group, accelerated tumor growth was measurable after week 7, and significantly larger tumors were present by the end of the study. This growth pattern could be explained by a small but persistent increase in the proliferative rate of tumor cells exposed to mevalonate over the course of the experiment. During the early weeks of tumor development, a small difference in proliferation would result in an indiscernable increase in tumor size compared with controls. Over a more extended time period, however, an increasing divergence in tumor size between the two groups became apparent. Our findings are in accordance with those of others who report similar patterns of growth in studies of promoters of MDA-MB-435 cell-derived tumors in nude mice (34, 35). To investigate whether increased proliferation may, indeed, mediate the growth-promoting properties of mevalonate, we next studied the effects of mevalonate on the same cancer cells in vitro.

Concentrations of mevalonate used in cell culture experiments were designed to amplify physiologically relevant effects over a much shorter time period than required for tumor growth. Mevalonate at both 1 and 5 mM increased cell proliferation rates by more than 50% over a period of 60 h. Furthermore, mevalonate increased the proportion of cells in S phase by promoting passage through the G1 restriction point, as evident in the smaller proportion of cells in G0/G1. The proportion of cells in G2/M did not differ significantly between treatment groups and control. Similar effects of mevalonate on cell cycle distribution and proliferation have been observed previously in breast cancer cells (36) and lymphocytes (37), albeit at substansially higher concentrations of mevalonate (up to 23 mM). As might be expected, these effects are the converse of cell cycle events that follow mevalonate depletion in cultured cells treated with statins (2). It has been known for some time that a critical level of mevalonate or an as yet unidentified non-sterol metabolite of mevalonate is required for initiation of DNA synthesis (2). Inadequate intracellular levels of mevalonate prevent entry of cells into S phase, resulting in a characteristic G1 phase growth arrest (2, 38).

The G1 arrest that results when mevalonate is depleted by statins in cultured cells is associated with changes in several regulators of cell cycle events that follow mevalonate depletion in cultured cells treated with statins (2). It has been known for some time that a critical level of mevalonate or an as yet unidentified non-sterol metabolite of mevalonate is required for initiation of DNA synthesis (2). Inadequate intracellular levels of mevalonate prevent entry of cells into S phase, resulting in a characteristic G1 phase growth arrest (2, 38).

The G1 arrest that results when mevalonate is depleted by statins in cultured cells is associated with changes in several regulators of cell cycle progression but most consistently with an inhibition of CDK-2 (31, 39, 40). CDK-2 controls initiation of DNA synthesis and replication of the chromosomes and is a major regulator of transition through the G1/S restriction point (for reviews see Refs. 41–43). Overexpression of the CDK-2 activator cyclin E in fibroblasts prevents cell cycle arrest following mevalonate depletion by lovastatin (39), suggesting that CDK-2 is an important regulator of the effects of mevalonate on G1/S phase progression. In the present study, mevalonate increased total CDK-2 activity as determined by its ability to phosphorylate histone H1. Enhanced CDK-2 activity was also evident in immunoprecipitates of cyclin A, indicating that mevalonate increased kinase binding to this activating subunit.
Mevalonate treatment resulted in little change in histone H1 kinase activity associated with cyclin E, suggesting that increased activation of CDK-2 by cyclin A was primarily responsible for the increased total kinase activity evident in cellular extracts. Elevated mevalonate also decreased expression of the CDKIs p21Cip1 and p27Kip1 and reduced the inhibitory binding of p21Cip1 to CDK-2 (31). Low expression levels of p27Kip1 precluded its detection in immunoprecipitates of CDK-2. Both p21Cip1 and p27Kip1 are regulators of the G1 restriction point and of CDK-2 activity (44, 45). Finally, we have shown that although total expression levels of CDK-2 did not change with mevalonate treatment, there were increased levels of the 33-kDa active form of CDK-2 that had been phosphorylated on its threonine 160 residue. Taken together, our results indicate that mevalonate increases CDK-2 activity through effects at multiple levels of regulation that are effectively the converse of events observed in mevalonate-depleted cells (31, 39, 40, 46). Increased CDK-2 activity may explain, at least in part, the growth-promoting effects of mevalonate on the cancer cells.

Our in vitro experiments strongly suggest that mevalonate promoted the growth of tumors in vivo, at least in part, through enhanced proliferation. It is possible, however, that mevalonate may also have altered the biology of either the host mouse or the tumor to enhance growth indirectly. Because mice used in this study were athymic, immunomodulation is an unlikely mediator of the effects we observed. Mevalonate may have promoted growth through effects on tumor vasculature. Neovascularization is critical to the expansion of tumors beyond ±1 mm in diameter (47). We have shown, however, that mevalonate at concentrations as high as 1 mM has no effect on growth of colon tumors in rats (52) and mice (53), in keeping with its ability to inhibit HMG-CoA reductase in intestinal cells (49, 54). A reduced risk of colorectal cancer has also been reported in one human trial of pravastatin (16).

In contrast to pravastatin, the lipophilic statins, including lovastatin, simvastatin, atorvastatin, and fluvastatin, inhibit HMG-CoA reductase activity equally well in cultured human hepatocytes and cells of extrahepatic origin (55). Although oral administration leads to efficient metabolism of these compounds by the liver, active metabolites can be found in the circulation (19). Moreover, orally administeredLovastatin and simvastatin have both been demonstrated to inhibit cholesterol synthesis in a number of extrahepatic tissues in mice (49). Thus, as demonstrated previously in mononuclear leukocytes (56) and in contrast to pravastatin, the lipophilic statins would be able to mitigate the increase in reductase that accompanies cholesterol reduction therapy through direct interaction with extrahepatic cells. This may explain why the use of simvastatin has been associated with a reduction in overall risk of cancer (22) and why the use of lovastatin has been shown to significantly lower the risk of melanoma (23). Furthermore, studies in rodents show a protective effect of lipophilic statins on the growth of several diverse tumor types (57–65), likely by decreasing mevalonate synthesis.

In summary, we report that mevalonate promotes the growth of tumors derived from human cancer cells grown in nude mice. As confirmation of these observations, the cells treated with mevalonate in culture demonstrated increased proliferation with accelerated entry into S phase. Furthermore, these cells had enhanced total CDK-2 activity, enhanced CDK-2 activity in immunoprecipitates of cyclin A, increased activating phosphorylation of CDK-2, and decreased inhibitory binding of p21Cip1 to CDK-2. Our findings demonstrate that mevalonate promotes tumor growth, and this may explain the elevated risk of cancer associated with cholesterol-lowering therapy in some studies.

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Note Added in Proof—A paper published after our manuscript was submitted indicates that the MDA-MB-435 cancer cells utilized in the present study most likely originated from breast epithelium but have undergone dedifferentiation during tumor progression to a melanocyte phenotype as a result of genetic instability (Sellappan, S., Grijalva, R., Zhou, X., Yang, W., Eli, M. B., Mills, G. B., and Yu, D. (2004) Cancer Res. 64, 3479–3485).

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Mevalonate Promotes the Growth of Tumors Derived from Human Cancer Cells \textit{in Vivo} and Stimulates Proliferation \textit{in Vitro} with Enhanced Cyclin-dependent Kinase-2 Activity

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