Evidence for Isopentenyladenine Modification on a Cell Cycle-regulated Protein*

Jerry R. Fausť and J. Fred Dice

From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

(Received for publication, November 9, 1990)

We have prepared antibodies that recognize isopentenyladenosine (i'A), a modified nucleoside derived from mevalonic acid (MVA). In immunoblot assays, affinity-purified anti-i'A antibodies specifically bound to a 26-kDa protein (i'A26) in Chinese hamster ovary cells. Anti-i'A recognition of i'A26 was blocked with i'A but not adenosine or isopentenol. Employing immunoblot analysis we have quantitated the level of i'A26 in cells expressing various rates of DNA synthesis. The cellular content of i'A26 was reduced 4-fold in quiescent cells cultured in the absence of serum. When serum-deprived cells were stimulated to enter the cell cycle, the amount of i'A26 increased in the cells during the G1 phase. However, when synchronized cells were stimulated with serum-containing medium in the presence of mevinolin (an inhibitor of cellular MVA synthesis), we observed impaired G1 expression of i'A26 and delayed onset of S phase DNA synthesis. Mevinolin addition to asynchronously growing cells resulted in low rates of cellular DNA synthesis and suppressed levels of i'A26 which were reversed by coincubation with MVA. The ability of MVA to restore DNA synthesis and the cellular content of i'A26 in mevinolin-treated cells showed similar MVA concentration and time dependencies. Regenerating liver tissue also exhibited elevated levels of i'A26. Thus, the expression of i'A26 correlates with cellular proliferation and time and may be induced by i'A26 contains isopentenyladenosine moieties and mediates isoprenoid regulation of DNA synthesis. Isopentenyladenylated proteins may also function in cytokinin regulation of proliferation and differentiation in plants.

Isoprenoids such as cholesterol, dolichol, and ubiquinone are essential components in a wide variety of mammalian cellular processes. MVA is the central precursor for all cellular isoprenoids, and adequate levels of MVA are required for sustained cell growth (1). Employing both pharmacological (2–8) and genetic (9) manipulations, many reports have documented that MVA is also acutely required for DNA replication. No clear evidence has emerged implicating either cholesterol, dolichol, or ubiquinone in acutely regulating DNA synthesis, and the identity of the cellular isoprenoid required for DNA replication is unknown.

Recently much attention has focused upon isoprenylated proteins, i.e. macromolecules that are radiolabeled when cells are incubated with radioactive MVA (6, 9–15). Consistent with a potential regulatory role in cell proliferation, some isoprenylated proteins are localized in nuclear fractions (13, 16). Also, Sinensky and Logel (9) observed a correlation between the decline in DNA synthesis in cells deprived of MVA and the rate of turnover of MVA-derived radioactivity in certain isoprenylated proteins.

Cellular ras and nuclear lamins are two types of cellular proteins which have been shown to contain isoprenyl groups (15). In ras, the carboxy-terminal cysteine contains a farnesyl moiety (Fig. 1B) covalently attached by a thioether linkage (17, 18). Farnesylation is required for proteolytic processing, palmitoylation, and tight binding of ras to cellular membranes (17, 19–21). In the absence of farnesylation, oncogenic forms of ras do not transform cultured cells (17–20). Lamin B also contains farnesyl residues covalently attached to cysteine (22–25). Farnesylation may promote binding of lamin B to the inner nuclear membrane and contribute to the integrity of the nuclear matrix. Also, lamin A is post-translationally modified by an isoprenyl moiety (26). Although the exact chemical nature of the modification has not been elucidated, i′A contains an amino acid motif, identical to that in ras and lamin B, which is required for farnesylation (25). Isoprenylation of lamin A is required for proteolytic processing of the precursor to mature lamin A (26). Geranylgeranyl (Fig. 1B) is another isoprenyl modification occurring in mammalian cell proteins (27, 28). Given the presumed function of ras in regulating cell growth and the structural role of lamins in nuclear architecture, speculation has emerged that either of these isoprenylated proteins may regulate cell proliferation.

Isopentenyladenosine (i′A) is a modified base found in tRNA from a wide variety of eukaryotic and prokaryotic cells (29). The i′A moiety (Fig. 1A) is derived from 3-methyl-2-buten-1-yl pyrophosphate, an intermediate in cellular isoprenoid biosynthesis. Isopentenyladenosine is found adjacent to the 3′ end of the anticodon of tRNAs. This modified nucleoside appears only in tRNAs that bind to codons containing uridine as the first base. In bacteria, isopentenylated tRNAs have been implicated in the regulation of aromatic acid uptake (30) and aerobiosis (31). The precise role of the modified nucleotide in tRNA metabolism of eukaryotes is unknown.

Free, non-tRNA-associated i′A has been observed in the yeast strains Saccharomyces cerevisiae and Schizosaccher-
protein may correlate with rates of DNA synthesis, we might expect that the cellular content of the modified gene expression, and differentiation in various tissues synthetic pathway independent of isopentenylated tRNA deg-
result in severely reduced amounts of isopentenylated tRNAs. This latter result indicates that free i6A may be derived via a synthetic pathway independent of isopentenylated tRNAs. In plants, i6A and related compounds are collectively referred to as cytokinins (33). Cytokinins are an important class of plant growth hormones which regulate cell division, gene expression, and differentiation in various tissues (34).

If an isoprenylated protein regulates DNA synthesis then we might expect that the cellular content of the modified protein may correlate with rates of DNA synthesis, i.e. the level of the isoprenylated protein would be low in cells expressing reduced rates of DNA replication resulting from MVA deprivation. Antibodies recognizing an isoprenylated protein could be used to quantitate the level of the modified protein in immunoblots performed on cell extracts. Within the context of MVA metabolism, we felt that the most meaningful quantitation would be achieved by using antibodies that are specific for the isoprenyl moiety in the modified protein. Knowing that adenylation and ADP ribosylation are antecedent nitrocellulose strips were incubated with sera or affinity-puri-
myces pombe (32). The cellular level of free i6A was not decreased in defective yeast strains possessing mutations that result in severely reduced amounts of isopentenylated tRNAs. This latter result indicates that free i6A may be derived via a synthetic pathway independent of isopentenylated tRNA deg-

Antibodies recognizing an isoprenylated protein could be used to quantitate the level of the modified protein in immunoblots performed on cell extracts. Within the context of MVA metabolism, we felt that the most meaningful quantitation would be achieved by using antibodies that are specific for the isoprenyl moiety in the modified protein. Knowing that adenylation and ADP ribosylation are antecedent nitrocellulose strips were incubated with sera or affinity-puri-

EXPERIMENTAL PROCEDURES

Buffers—Buffer A is 10 mM Tris chloride, 140 mM NaCl, 0.02% (w/v) sodium azide, pH 7.5. Buffer B is 50 mM Tris chloride, 1 mM EDTA, 250 mM sucrose, pH 7.4. Buffer C is 20 mM Tris chloride, 500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4. Buffer D is buffer C containing 5% (w/v) Carnation nonfat powdered milk. Tris/saline is 50 mM Tris chloride, 155 mM NaCl, pH 7.4. Gel loading buffer is 62.5 mM Tris chloride, 2.7 M urea, 5% (w/v) sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (added fresh daily), 10% (v/v) glycerol, 20 mg/ml bromophenol blue, pH 6.8. Buffer E is buffer D containing 10 mg/ml BSA

Materials—The following chemicals were obtained from Aldrich: isopentenyladenosine hemihydrate, ethylene glycol, sodium borohydride, formic acid, sodium iodate, and pyridine. Ovalbumin, sodium iodide, Freund's adjuvants, α-aminocetoxy agarose, ADP ribose, trans-

Cell Culture—Cultured cells were grown in a humidified incubator containing 5% CO₂ at 37°C. Stock cultures of CHO-K1 cells were grown in medium 1 (Ham's F-12 medium containing 5% (v/v) newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 25 mM Hepes, pH 7.4). Cells were seeded on day 0 at 100,000 cells/100-mm² dish in 8 ml of medium 1. On day 2, experiments were initiated as indicated.

Preparation of i6A Conjugated to BSA, Immunization of Rabbits, and Affinity Purification of Anti-i6A Antibodies—Antibodies recognizing i6A were prepared by immunizing rabbits with i6A-BSA and affinity purifying the antisera on i6A-Sepharose. i6A-BSA was prepared at room temperature essentially as described by Erlanger and Beiser (30). Briefly, 100 μg of i6A was stained for 20 min in 5 ml of 0.1 M sodium iodate. Excess iodate was decomposed by the addition of 0.3 ml of 1 M ethylene glycol. After 5 min the reaction mixture was added to a stirring aqueous solution (10 ml) of 280 mg of BSA (Fraction V from Pentex) adjusted to pH 9.3 with 5% (w/v) potassium carbonate. The reaction was adjusted for 45 min while maintaining pH 9.0-9.5 with 5% (w/v) potassium carbonate. Finally, the solution was dialyzed for 24 h against 6 liters of deionized water. The preparation yielded 144 mg of protein in 39 ml. The i6A concentration (as measured by absorbance at 280 nm) was 0.57 μg/ml.

As proposed by Erlanger and Beiser (30), periodate oxidation of i6A would cleave the ribose ring structure between carbons 2 and 3. In alkaline solution, the resulting diahyde would react with a primary amine on BSA, forming a cyclic hemiacetal. Subsequent borohydride reduction produces a stable morpholine derivative in which a new six-member ring would form consisting of the original five atoms from the ribose ring plus the amino nitrogen attached to BSA. Thus, the attachment of i6A to the protein is via the ribose moiety and should not alter the atomic structure of isopentenyladenine. Two New Zealand White rabbits were each immunized with 2 mg of i6A-BSA emulsified with Freund's complete adjuvant on day 0. The rabbits received subsequent injections of 2 mg of i6A-BSA diluted 1:1 with incomplete Freund's adjuvant on days 8 and 21. On day 53 the rabbits were killed, and immune serum was isolated after clotting. The serum was stored at −70°C.

Coupling of i6A to AH-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) was exactly as described (37). For affinity purification, one aliquot (8 μl) of immune serum was diluted to 30 ml with buffer A and precleared by incubation with 10 ml of ω-aminocetoxy agarose (2 h, room temperature) followed by centrifugation (1,000 × g, 10 min, room temperature). All subsequent procedures were performed at 4°C. The precleared supernatant was mixed with 5 ml of i6A-Sepharose and agitated gently overnight. Next the slurry was percolated through a 10 × 100 mm plastic column. The column was washed with buffer A (75–100 ml) until absorbance at 280 nm was less than 0.06. Two fractions of anti-i6A antibodies were eluted from the column. Fraction 1 was eluted with 20 ml of buffer A containing 500 mM instead of 140 mM NaCl. Subsequently, fraction 2 was eluted with 20 ml of 10% (v/v) pyridine in buffer A. Both fractions were dialyzed against 6 liters of buffer A and stored at −70°C.

Dot Blotting—Serial dilutions of i6A-BSA and BSA were made in 10 μl of 10% (v/v) Carnation nonfat powdered milk, and aliquote (200 μl) were spotted onto nitrocellulose (Schleicher & Schuell) in a dot blotting manifold. After applying vacuum to deposit the proteins onto nitrocellulose, the sheet was cut vertically between the rows of dots producing replicate strips of nitrocellulose containing various amounts of i6A-BSA and BSA. The replicate strips were hybridized with buffer C and 32P-labeled i6A-BSA and buffer D as described in the immunoblotting procedure below. Replicate nitrocellulose strips were incubated with sera or affinity-puri-
fied anti-i'A antibodies in 3 ml of buffer D on a rotating platform for 3.5 h at room temperature. The strips were then pooled together and washed as described in the immunoblotting procedure below. Next the nitrocellulose strips were incubated with a 1:2,000 dilution of alkaline phosphatase–linked goat anti-rabbit antiserum for 2 h at room temperature on a rotating platform and washed again. The alkaline phosphatase–linked goat antiserum was prepared in 100 mM sodium bicarbonate, 1 mM magnesium chloride, pH 9.8, using the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, as recommended by the supplier (Promega Biotec, Madison, WI).

**Measuring Cell Protein and DNA Synthesis**—As indicated in the figure legends, monolayers were labeled with [3H]thymidine in a 37 °C incubator. The labeled monolayers four times with 10 ml of Tris/saline: once quickly, twice precipitated with 1.5 ml of 15% (w/v) trichloroacetic acid (15 min, 15,000 rpm, 5 min, 4 °C). These cell pellets were stored at −70 °C. During the recovery phase the rats were allowed free access to food and water. On day 3 the rats were killed, and liver tissues (designated “regenerating”) were removed and stored at −70 °C. Aliquots of liver were thawed on ice in the presence of 14 volumes of buffer B. The tissue was Dounce homogenized using 20 strokes with a loose pestle followed by 20 strokes with a tight fitting pestle. The homogenate was centrifuged (1,000 × g, 10 min, 4 °C) and the supernatant saved. The pellet was resuspended in 1 volume of buffer B by Dounce homogenization 20 times with a tight fitting pestle. After centrifugation of the homogenate (1,000 × g, 10 min, 4 °C), the resulting supernatant was combined with the initial supernatant. The pooled supernatants were centrifuged (100,000 × g, 60 min, 4 °C) and stored at −70 °C.

**RESULTS**

**Presence of Anti-i'A Antibodies in Immune Serum and Isolation of Affinity-purified Antibodies**—We prepared antibodies directed against i'A by immunizing rabbits with i'A linked to bovine serum albumin (i'A-BSA). Affinity-purified anti-i'A antibodies were obtained by fractionation of the immune serum with i'A linked to Sepharose (i'A-Sepharose). Using a dot blot assay, we screened for the presence of anti-i'A antibodies in the sera and affinity-purified fractions. The dot blots were prepared by spotting various amounts of either bovine serum albumin (BSA) or i'A-BSA onto nitrocellulose in multiple vertical rows. Replicate strips were obtained by vertically cutting the nitrocellulose between the rows of dots. These replicate strips were used to evaluate the presence of anti-i'A antibodies in test solutions. Antibody binding to the immobilized proteins was detected with alkaline phosphatase conjugated to goat anti-rabbit immunoglobulins and subsequent color reaction. By analyzing the degree of the color reaction formed over the various amounts of i'A and i'A-BSA, we evaluated the relative concentration of antibodies in the test solutions.

Fig. 2 shows that antibodies binding to either BSA or i'A-BSA were not detected in preimmune rabbit serum (strip 1).

**Preparation of Control and Regenerating Rat Liver Supernatants**—On day 0 male Sprague-Dawley rats (100–150 g) were anesthetized, and an approximately 70% hepatectomy was performed. The liver tissue removed at this time (designated “control”) was stored at −70 °C. During the recovery phase the rats were allowed free access to food and water. On day 2 the rats were killed, and liver tissue (designated “regenerating”) was removed and stored at −70 °C. Aliquots of liver were thawed on ice in the presence of 14 volumes of buffer B. The tissue was Dounce homogenized using 20 strokes with a loose pestle followed by 20 strokes with a tight fitting pestle. The homogenate was centrifuged (1,000 × g, 10 min, 4 °C) and the supernatant saved. The pellet was resuspended in 1 volume of buffer B by Dounce homogenization 20 times with a tight fitting pestle. After centrifugation of the homogenate (1,000 × g, 10 min, 4 °C), the resulting supernatant was combined with the initial supernatant. The pooled supernatants were centrifuged (100,000 × g, 60 min, 4 °C) and stored at −70 °C.
However, immune serum contained antibodies that bound to both proteins (strips 2–4). Note that antibody recognition can be detected on spots containing 4 ng of \( \text{iiA-BSA} \) whereas at least 64 ng of BSA was required to visualize antibody binding. This result indicates that the immune rabbit serum had a higher titer of antibodies directed against \( \text{iiA-BSA} \) relative to BSA. Purification of the immune serum with \( \text{iA-Sepharose} \) resulted in a substantial reduction in the concentration of anti-BSA binding antibodies (strips 5–7). Also, the concentration of anti-iiA-BSA antibodies did change appreciably as a result of the affinity purification procedure. This observation implies that the affinity-purified fractions contain antibodies that recognize iiA specifically.

**Immunoblotting of CHO Cell Extracts with Affinity-purified Anti-iiA Antibodies**—Unfractionated CHO cells were analyzed by immunoblotting for macromolecules that bind affinity-purified anti-iiA antibodies (Fig. 3). As shown in lane 1, CHO cells growing in serum-containing medium contain two macromolecules (approximate molecular sizes, 56 and 26 kDa) that bind 125I-protein A after incubation with anti-iiA antibodies. No autoradiographic bands were observed when affinity-purified anti-iiA antibodies were omitted from the immunoblotting procedure (data not shown). Also, when unfractionated immune serum was used in the immunoblotting procedure, we observed antibody binding to both the 26- and 56-kDa macromolecules. Preimmune serum gave no antibody recognition at those molecular masses (data not shown). These results indicate that antibodies specifically recognizing these two entities were produced by the rabbit in response to immunization with iiA-BSA. Although two of two immunized rabbits produced antibodies specifically recognizing iiA-BSA, affinity-purified antibodies recognizing the 56- and 26-kDa macromolecules were present in only one rabbit serum (data not shown). Antibodies recognizing the 26-kDa macromolecule were observed exclusively in the 0.5 M NaCl eluate of the affinity purification (see “Experimental Procedures”). Antibodies recognizing the 56-kDa entity were present in both affinity-purified fractions; however, the pyridine-eluted fraction contained the highest titer (data not shown).

What is the chemical nature of these immunoreactive macromolecules? Several data indicate that the 26-kDa macromolecule is a protein. Knowing that isopentenyl tRNAs are cellular macromolecules that could bind anti-iiA antibodies, we subjected purified rat liver tRNA to immunoblotting analysis. When aliquots greater than 100 µg of liver tRNA were analyzed we observed a very diffuse autoradiographic image in the molecular mass range between 68 and 97 kDa (data not shown). In subcellular fractionation studies, the 26-kDa macromolecule was found to localize exclusively with the 100,000 X g supernatant, not with cellular DNA which sediments at low centrifugation forces (data not shown). Also, when the 100,000 X g supernatant was pretreated with trypsin and subsequently analyzed by immunoblotting, the 26-kDa immunoreactive macromolecule disappeared. Trypsin digestion of the 26-kDa macromolecule was prevented with soybean trypsin inhibitor (data not shown). Consequently, we have concluded that the 26-kDa macromolecule is a protein, and we will refer to it as iiA26.

Using immunoblotting analysis we have determined the relative concentration of the antibody-binding entities in CHO cells cultured in various media (Fig. 3). When CHO cells were incubated for 20 h with mevinolin, a potent inhibitor of MVA biosynthesis, the cellular content of iiA26 declined dramatically (lane 2). Incubation of mevinolin-treated CHO cells with MVA restored the cellular content of iiA26 to the uninhibited level (lane 3). These observations indicate that the mevinolin-induced suppression of iiA26 is specifically due to inhibition of cellular MVA synthesis. Also, CHO cells incubated for 31 h in serum-depleted medium have a lower level of iiA26 (lane 4) which was not reversed when serum-starved cells were incubated with exogenous MVA (lane 5). This indicates that reduced availability of MVA was not mediating the serum-deprived suppression. By densitometric scanning of multiple autoradiographic images in several different experiments we determined that incubation in either the presence of mevinolin or in the absence of serum, respectively, resulted in an average 4-fold decline in the cellular content of iiA26.

Fig. 3 also shows that the levels of the 56-kDa antibody-binding macromolecule did not vary in CHO cells preincubated in the various media. The invariant level of this macromolecule serves as a convenient control for equivalent loading of the gel and uniform immunoblotting.

**Competition for Antibody Binding to iiA26**—Based on our observations that the iiA26 binds antibodies present in iiA-BSA immune serum as well as fractions purified with iiA-Sepharose, we speculate that the 26-kDa protein contains isopentenyladenine moieties that are mediating the binding of anti-iiA antibodies. We tested for the presence of iiA-specific binding of antibodies by performing the immunoblotting incubations in the presence of potential competing agents (Fig. 4). Affinity-purified antibodies were preincubated with various amounts of iiA, adenosine, or 3-methyl-2-buten-1-ol (an alcohol derivative of the isoprenoid moiety in iiA). Then the antibodies were used to immunoblot replica strips of nitrocellulose containing resolved CHO cell proteins. To avoid the possibility that the competitors may differentially bind to BSA and nonfat dry milk, thus lowering the effective concentration of the competitors, the preincubation and primary antibody binding reactions were performed in the absence of...
Cell Cycle Regulation of an Isopentenyladenylated Protein

26 kDa were preincubated overnight at room temperature in 2 ml of buffer C containing 100 μl of dimethyl sulfoxide and the indicated concentrations. Washed, incubated with goat anti-rabbit antibodies linked to alkaline phosphatase, and visualized as described under “Experimental Procedures.” Sixty μl of fraction 1 affinity-purified antibodies was observed with 12 and 20 mM i'A (Fig. 3, lanes 1–6). We note that the time courses of mevinolin inhibition of DNA synthesis (panel A) and suppression of i'A26 (panel C, lanes 1–6) are very similar. When MVA was added to cells preincubated with mevinolin for 21 h, FA26 continued to decline for an additional 6 h (lanes 7 and 8). By 9 h after MVA addition the cellular content of the i'A26 had rebounded and continued to increase during the remainder of the time course (lanes 9–12). Thus, the time courses of MVA restoration of DNA synthesis (panel B) and cellular content of i'A26 (panel C, lanes 7–12) were similar.

Reversal of Mevinolin-inhibited DNA Synthesis Correlates with the Content of i'A26 in Cells Incubated with Various Concentrations of MVA—CHO cells were incubated in the absence or presence of mevinolin and various concentrations of MVA. After 20 h the cells were pulse labeled with [3H] thymidine to determine the rates of DNA synthesis (Fig. 6A). In this experiment, mevinolin suppressed DNA synthesis 85%. Addition of MVA to the culture medium reversed the mevinolin inhibition of DNA synthesis in a concentration-dependent manner with full restoration at 300 μM MVA. Greater than 300 μM MVA did not stimulate cellular DNA synthesis further.

Fig. 6B shows the result of immunoblotting with affinity-purified anti-i'A antibodies on the same cells pretreated with mevinolin and various concentrations of MVA. Low concentrations of MVA partially prevented mevinolin suppression of the cellular i'A26. Full restoration occurred at 300 μM MVA. Greater than 300 μM MVA did not induce increased levels of i'A26 in CHO cells.

Mevinolin Inhibition of S Phase DNA Synthesis and Impaired Expression of i'A26 in Synchronized CHO Cells—As noted above, CHO cells cultured in serum-deprived medium have low levels of i'A26 (Fig. 3, lane 4) which are not restored to normal by the addition of MVA (Fig. 3, lane 5). This result implies that cells cultured in low concentrations of serum are not compromised with respect to MVA. We speculate that the low cellular content of the i'A26 may be related to the quiescent state (G0) of CHO cells cultured in low serum medium. When quiescent cells are refed medium containing a high concentration of serum, the cells enter the cell cycle in synchrony. The synchronous progression of the cells to S phase

![Fig. 4. Competition for immunoblotting.](image-url)
of the cell cycle can be monitored by pulse labeling cells with [\textsuperscript{3}H]thymidine and quantitating DNA synthesis. We were interested in determining at which stage in the cell cycle \(i^A26\) appears when quiescent cells are stimulated. Also, we attempted to correlate the appearance of \(i^A26\) with S phase DNA synthesis (Fig. 7).

CHO cells were preincubated in low serum medium and subsequently refed high serum medium in the absence and presence of mevinolin. In panel A, the cells' progression to S phase was determined by measuring the rate of cellular DNA synthesis. Increased rates of DNA synthesis were apparent in cells 9 h after the addition of serum-containing medium, and peak levels of replication were observed in the cells at the 15-h time point. When quiescent CHO cells were stimulated with serum-supplemented medium plus mevinolin, S phase DNA synthesis was dramatically inhibited.

Panel B shows the cellular levels of \(i^A26\) during cell cycle progression. In the absence of mevinolin (lanes 2-6) increased levels of the \(i^A26\) were apparent in cells within 6 h after the addition of serum. The cellular content of \(i^A26\) rose further with increasing time in the cell cycle. When cells were stimulated with serum in the presence of mevinolin (lanes 7-11), the amount of \(i^A26\) remained low. It is only after 15 h of serum stimulation in the presence of mevinolin that we observed an increase in the cellular content of this protein. Thus, S phase DNA synthesis is preceded by an increase in the cellular level of \(i^A26\). Also, mevinolin inhibition of S phase DNA synthesis correlates with the lack of induction of \(i^A26\).

**Elevated Expression of \(i^A26\) in Regenerating Rat Liver—**

Normal rat liver exhibits low rates of DNA synthesis and cell division. However, partial hepatectomy dramatically stimulates the remaining liver cells to proliferate (40). By 48 h posthepatectomy, the liver mass has regenerated to its normal size. By comparing unstimulated \textit{versus} regenerating liver, cell proliferation can be studied in an intact tissue. We sought to determine if \(i^A26\) was present in liver and whether the expression of this macromolecule correlates with liver cell proliferation.

Immunoblots were performed on 100,000 X g supernatants from unstimulated (lanes 2 and 4) and regenerating (lanes 3 and 5) liver obtained in two separate experiments (Fig. 8). A sample of unfractionated growing CHO cells (lane 1) was included in this analysis. We observed that regenerating liver supernatant has several immunoreactive bands. Quantitatively, antibody recognition is greatest for macromolecules migrating with apparent molecular sizes of 56, 39, and 26 kDa. Although the content of the 56- and 39-kDa macromolecules was unchanged in the unstimulated (control) liver samples, the expression of the \(i^A26\) was greatly decreased.

**DISCUSSION**

A major focus of these studies is the identification of a protein whose expression in cells correlated with cell proliferation. Five experiments substantiate the relationship between the cellular content of \(i^A26\) and cellular DNA synthesis. First, mevinolin induced parallel declines in the cellular content of \(i^A26\) and rates of DNA synthesis (Fig. 5). Second, the time course of MVA restoration of mevinolin-inhibited DNA synthesis displayed a prominent lag phase. A similar
lag was also observed in the expression of i'A26 (Fig. 5). Third, a strong correlation exists between the concentrations of MVA which prevent both mevinolin-inhibited DNA synthesis and mevinolin suppression of i'A26 (Fig. 6). Fourth, during synchronous progression of cells in the cell cycle, increased expression of i'A26 occurred in the G1 stage of the cell cycle, preceding the cells entry into the DNA replicative phase (Fig. 7). Synchronized cells incubated with mevinolin, which impairs their transit to S phase, showed delayed expression of i'A26 (Fig. 7). Fifth, proliferating liver tissue contains elevated amounts of i'A26 when compared with quiescent liver (Fig. 8).

This paper describes two types of experimental evidence indicating that i'A26 contains isopentenyladenine: immunologic and metabolic. First, antibodies that recognize i'A26 are not present in the preimmune serum but are found in serum of the rabbit immunized with isopentenyladenine linked to BSA. Furthermore, the specific antibodies that recognize i'A26 also bind to isopentenyladenine linked to Sepharose. The competition experiment (Fig. 4) verified that an isopentenyladenosine-specific binding site on the antibodies is directly responsible for antibody recognition of i'A26 and suggests that the 26-kDa protein contains isopentenyladenine.

Second, isopentenyladenine is derived metabolically from MVA. Therefore, the synthesis of this modified base is suppressed in cells incubated with mevinolin. If an isopentenyladenine-containing protein was degraded in cells or the modification was removed, then the steady-state level of isopentenyladenine-containing protein would decline in mevinolin-treated cells. Indeed, we show (Figs. 3, 5, and 6) that mevinolin suppresses the cellular content of i'A26, and the mevinolin effect is reversed with MVA. Incubating cells in the absence of serum also suppresses the level of i'A26, and the modified protein reappears when the cells are refed serum-containing medium. Mevinolin (MVA deprivation) blocks the normal appearance of i'A26 in the synchronized cells (Fig. 7). Since the anti-i'A antibodies are recognizing a modification on the 26-kDa protein, we cannot distinguish whether the regulation of i'A26 is due to changes in the amount of the protein or in the proportion of the protein modified by isopentenyladenine.

At present we have been unsuccessful in attempting to immunoprecipitate i'A26 from cells radiolabeled with either
also observed antibody binding to several bands in the immunoblot of the liver supernatants (Fig. 8). Liver may contain additional isopentenyladenine-modified proteins not present in CHO cells. It is also possible that many cellular proteins contain isopentenyladenine moieties; however, our anti-i'A antibodies may recognize only a subset. Antibody binding to small epitopes may be influenced by various chemical structures, e.g. amino acid side chains, in the vicinity of the epitope.

We are unaware of any reports describing isopentenyladenine modification of proteins and have considered that i'A26 may not contain this new modification. Mevinolin suppression of i'A26 may be a consequence of the mevinolin-induced quiescent state in CHO cells and may not reflect decreased isopentenyladenine modification as a result of MVA deprivation. In fact, we show that cells rendered quiescent as a result of incubation in the absence of serum growth factors express reduced levels of i'A26. Also, in addition to i'A, our antibodies may promiscuously recognize another unknown modification or unique cluster of amino acids with a conformation similar to isopentenyladenine. Nevertheless, we speculate that i'A26 contains isopentenyladenine and that elevated expression of i'A26 requires both serum growth factors and MVA in concert.

Studies on the regulation of the mammalian cell cycle have shown that nuclei of cells in G1 phase will rapidly undergo DNA replication when the G1 cells are fused to S phase cells (41). The major conclusion from these experiments is that a limiting trans-acting inducer of S phase accumulates gradually throughout G1 and stimulates S phase when it reaches a critical threshold level. Also, Pardee and co-workers reviewed evidence (42, 43) that a specific labile protein must accumulate to a sufficient level (termed "restriction point") in order for G1 cells to traverse S phase. Our studies show that i'A26 accumulates in G1 phase prior to the onset of S phase. Therefore, i'A26 may participate in trans-induction and/or restriction point control of S phase.

Fairbanks et al. (4) studied the specific requirement for MVA during the cell cycle. By varying the time of addition and removal of both mevinolin and MVA to culture medium after platelet-derived growth factor stimulation, these investigators delineated a critical time period in G1 phase when synchronized human fibroblasts require MVA for S phase DNA synthesis. They concluded that a MVA metabolite is required in G1 for cells to enter S phase. We report in this paper that i'A26 accumulates in G1 phase of CHO cells. Mevinolin treatment of synchronized CHO cells inhibited G1 accumulation of i'A26 and coincidentally delayed the onset of S phase. Consequently, i'A26 is a candidate for the critical MVA metabolite required for S phase DNA replication.

If mevinolin inhibition of DNA synthesis is due to decreased availability of i'A for modification of the 26-kDa protein, we might expect that i'A, or an isopentenyladenine derivative, would replace MVA in restoring mevinolin-inhibited DNA replication. Two groups have reported regulation of mammalian cell proliferation by isopentenyladenine and i'A. Gallo et al. (44) observed that i'A either stimulated or inhibited DNA synthesis, cell transformation, and mitosis in phytohemagglutinin-incubated human lymphocytes. The opposing effects of i'A were highly dependent upon the concentration and time of addition of the nucleoside.

Also, Siperstein and colleagues (45, 46) studied the role of MVA in regulating S phase DNA synthesis in baby hamster kidney cells. These workers reported that isopentenyladenine and its 4'-hydroxylated derivative, zeatin, could substitute for MVA in restoring DNA replication in compactin-treated cells (compactin, similar to mevinolin, is an inhibitor of cellular
MVA synthesis). Isopentenyladenosine was ineffective. Restoration of DNA synthesis was only observed in baby hamster kidney cells incubated within a narrow concentration range of isopentenyladenine and only when the modified base was added to cells late in G1 phase.

Several groups have reported that isopentenyladenine and/or i6A did not prevent mevinolin-inhibited DNA synthesis (3, 7–9, 47). Apparently the ability of i6A to modulate DNA replication is highly dependent upon the concentration, time of addition, and chemical form of the isopentenyl derivative. Also, these isopentenyl compounds appear to have multiple effects in various cultured cells, i.e. inhibition of protein and RNA synthesis and stimulation/inhibition of DNA replication. Perhaps the disparate effects of the isopentenyladenine derivatives in various cells may be due to dissimilar metabolic capabilities of the specific cells. If a phosphorylated nucleotide form of i6A is responsible for modulating DNA synthesis, then the differential expression of purine salvage and catabolic pathways may explain the conflicting observations in various cell types incubated with either the modified base or nucleoside.

Cytokinins are i6A derivatives that serve as important plant hormones regulating cell division, gene expression, and differentiation in various tissues (48). Although isopentenyl tRNA degradation is a minor pathway contributing to i6A levels in plant tissue, plant cytokinins are mostly derived via direct modification of adenosine monophosphate. Direct evidence for the role of i6A in plant cell proliferation has come from studies on tumor induction by Agrobacterium tumefaciens in crown gall disease (49, 50). Crown gall disease is a result of the bacteria transferring a gene coding for i6A biosynthesis into plant cells which leads to increased production of the hormone. The elevated levels of cytokinin are specifically responsible for uncontrolled tumor growth as well as suppression of root growth. The molecular mechanisms underlying cytokinin-controlled differentiation and growth induction/suppression in plants are unknown. Perhaps isopentenyladenylated proteins mediate cytokinin regulation in plants. Conjugates of cytokinins and the amino acids alanine (51) and the 2-aminothicyric acid moiety of methionine (52, 53) have been identified in soybean and Dicyostelium discoideum, respectively. It is possible that these cytokinin-conjugated amino acids may arise from degradation of isopentenyladenylated proteins. Our present observations correlating the concentration of an anti-i6A-binding cellular component with mammalian cell replication may underscore a common mechanism controlling proliferation in both the animal and plant kingdoms.

Acknowledgments—We thank Zenaida Gatmaitan and Rosana Kapper of this department for assistance with the regenerating rat liver experiments; Laura J. Terleckey for preparation of tissue culture pellets of this department for assistance with the regenerating rat liver.

REFERENCES
1. Brown, M. S., and Goldstein, J. L. (1980) J. Lipid Res. 21, 505–517
2. Habenicht, A. J. R., Glomset, J. A., and Ross, R. (1980) J. Biol. Chem. 255, 5134–5140
3. Larson, R. A., Chung, J., Scanu, A. M., and Yachnin, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3028–3032
4. Fairbanks, K. P., Witte, L. D., and Goodman, D. S. (1984) J. Biol. Chem. 259, 1546–1551
5. Siperstein, M. D. (1984) J. Lipid Res. 25, 1462–1468
6. Maltese, W. A., and Sheridan, K. M. (1987) J. Cell. Physiol. 133, 471–481
7. Langen, T. J., and Volpe, J. J. (1987) J. Neurochem. 49, 513–521
8. Doyle, J. W., and Kandutsch, A. A. (1988) J. Cell. Physiol. 137, 134–140
9. Sinensky, M., and Logel, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3257–3261
10. Schmidt, R. A., Schneider, C. J., and Glomset, J. A. (1984) J. Biol. Chem. 259, 10175–10180
11. Breung, E., and Rilling, H. C. (1986) Biochem. Biophys. Res. Commun. 139, 209–214
12. Faust, J., and Krieger, M. (1987) J. Biol. Chem. 262, 1996–2004
13. Maltese, W. A., and Erdman, R. A. (1989) J. Biol. Chem. 264, 18168–18172
14. Repko, E. M., and Maltese, W. A. (1989) J. Biol. Chem. 264, 9945–9952
15. Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) Trends Biochem. Sci. 15, 139–142
16. Sepp-Lorenzino, N., Azrulan, N., and Coleman, P. S. (1989) FEBS Lett. 245, 110–116
17. Casey, P. J., Solski, P. A., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323–8327
18. Jackson, J. H., Cochrane, C. G., Bourne, J. R., Solski, P. A., Buss, J. E., and Der, C. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3942–3946
19. Schafer, W. R., Kim, R. R. S., Thorner, J., Kim, S.-H., and Rine, J. (1989) Science 245, 379–385
20. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167–1177
21. Leonard, S., Beck, L., and Sinensky, M. (1990) J. Biol. Chem. 266, 5157–5160
22. Beck, L. A., Hosick, T. J., and Sinensky, M. (1988) J. Cell Biol. 107, 1307–1316
23. Wolda, S. L., and Glomset, J. A. (1988). J. Biol. Chem. 263, 5997–6000
24. Farnsworth, C. C., Wolda, S. L., Gelb, M. H., and Glomset, J. A. (1989) J. Biol. Chem. 264, 20422–20429
25. Vorbar, K., Kitten, C. T., and Nigg, E. A. (1989) EMBO J. 8, 4007–4013
26. Beck, L. A., Hosick, T. J., and Sinensky, M. (1990) J. Cell Biol. 110, 1489–1498
27. Farnsworth, C. C., Gelb, M. H., and Glomset, J. A. (1990) Science 247, 320–322
28. Rilling, H. C., Breung, E., Epstein, W. W., and Crain, P. F. (1990) Science 247, 318–320
29. Bjork, G. R., Ericson, J. U., Gustafsson, C. E. D., Hagervall, T. G., Jonsson, Y. H., and Wikstrom, P. M. (1987) Annu. Rev. Biochem. 56, 263–287
30. Buck, M., and Griffiths, E. (1981) Nucleic Acids Res. 9, 401–414
31. Buck, M., and Ames, B. N. (1984) Cell 36, 523–531
32. Laten, H. M., and Zahareas-Doktor, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1113–1115
33. Leeflang, P. S., and Palini, L. M. S. (1983) Annu. Rev. Plant Physiol. 34, 163–197
34. Skoog, F., and Armstrong, D. J. (1970) Annu. Rev. Plant Physiol. 21, 359–384
35. Wold, F. (1981) Annu. Rev. Biochem. 50, 783–814
36. Elranger, B. F., and Beiser, S. M. (1964) Proc. Natl. Acad. Sci. U. S. A. 52, 68–74
37. Humayun, M. Z., and Jacob, T. M. (1974) Biochim. Biophys. Acta 349, 84–95
38. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
39. Lamml, U. K. (1970) Nature 227, 680–685
40. Bucher, N. L. R., Patel, U., and Cohen, S. (1977) Adv. Enzyme Regul. 16, 205–213
41. Cross, F., Roberts, J., and Weintraub, H. (1989) Annu. Rev. Biochem. 58, 341–395
42. Pardee, A. B., Dubrow, R. H., Manolin, J. L., and Kletzien, R. (1978) Annu. Rev. Biochem. 47, 715–750
43. Pardee, A. B. (1980) Science 246, 603–608
44. Gallo, R. C., Whang-Peng, J., and Perry, S. (1969) Science 200, 400–402
45. Hunevees, V. Q., Wiley, M. H., and Siperstein, M. D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5842–5846
46. Quinsey-Huneveees, V., Galick, H. A., Siperstein, M. D., Erickson, S. K., Spencer, T. A., and Nelson, J. A. (1983) J. Biol. Chem. 258, 378–385
Cell Cycle Regulation of an Isopentenyladenylated Protein

47. Perkins, S. L., Ledin, S. F., and Studds, J. D. (1982) Biochim. Biophys. Acta 711, 83–89
48. Hall, R. H. (1973) Annu. Rev. Plant Physiol. 24, 415–444
49. Akiyoshi, D. E., Klee, H., Amasino, R. M., Nester, E. W., and Gordon, M. P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5994–5998
50. Weiler, E. W., and Schroder, J. (1987) Trends Biochem. Sci. 12, 271–275
51. Zhang, R., Letham, D. S., Wong, O. C., Nooden, L. D., and Parker, C. W. (1987) Plant Physiol. 83, 334–340
52. Abe, H., Uchiyama, M., Tanaka, Y., and Saito, H. (1976) Tetrahedron Lett. 42, 3807–3810
53. Ihara, M., Taya, Y., and Nishimura, S. (1980) Exp. Cell Res. 126, 273–278
54. Maltese, W. A., Sheridan, K. M., Repko, E. M., and Erdman, R. A. (1990) J. Biol. Chem. 265, 2148–2155
55. Maltese, W. A., and Sheridan, K. M. (1990) J. Biol. Chem. 265, 17883–17890