The Isolation and Characterization of Polycyclic Hydrocarbon-binding Proteins from Mouse Liver and Skin Cytosols

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SUMMARY

The major protein to which metabolites of methylcholanthrene are covalently bound has been purified from C3H mouse liver cytosol. Its properties are identical to the mouse skin h-protein, which may be a primary target of carcinogenic hydrocarbon metabolites during transformation to cancer. It has a molecular weight of 44,000, consists of 2 subunits of M.W. 20,000, has an isoelectric point (pI) of 8.05 to 8.6, and a sedimentation coefficient of 3.6 S. These physical properties are rather similar to those of ligandin, a hepatic protein that binds carcinogen metabolites, steroid anionic metabolites, bilirubin, and exogenous organic anions, but not to those of the rat liver azo dye carcinogen binding "slow h2-5S" protein. The h-protein and ligandin consistently give different pI values. Two minor basic proteins (molecular weights around 44,000 each), to which methylcholanthrene metabolites are covalently bound, have been separated from the h-protein by carboxymethyl-cellulose chromatography. Preliminary results indicate that these 2 minor proteins are related to ligandin. A protein to which methylcholanthrene is noncovalently bound was also identified in the acidic fraction of the mouse liver and skin cytosols and has been partially purified and characterized. It has a molecular weight of 60,000, a pI of 5.0, and a sedimentation coefficient of 4.5 S.

INTRODUCTION

The covalent binding of carcinogens to cytoplasmic proteins of target tissues (1, 6, 9, 12, 21, 29, 33, 37) and transformable cells in culture (13, 14) has been repeatedly demonstrated. These proteins, which have basic pI values, belong to the class of the "h" proteins (38). Ketterer et al. (10–12) isolated 2 azo dye-bound proteins from rat liver with molecular weights of 45,000 and 14,000, respectively. The former protein has been termed "ligandin" because of its wide range of binding affinities towards carcinogen metabolites, anionic steroid metabolites, bilirubin, and a number of organic anions (2, 12, 16–18, 22, 25, 26, 31). Sorof et al. (34–36) also isolated and purified a rat liver protein referred to as "slow h2-5S," which has the highest affinity of binding of all proteins for azo dye carcinogens.

Earlier work in this laboratory (1) established that carcinogenic hydrocarbons bind covalently to a specific protein from the cytosol of topically treated mouse skin. Noncarcinogenic hydrocarbons bind much less to this protein, which has been termed the h-protein due to its electrophoretic resemblance to the liver protein characterized by Sorof et al. (33). The mouse skin h-protein has similar properties to ligandin (17) but not to the slow h2-5S azoprotein (34, 35).

The origin, nature, and function (if any) of the various protein-carcinogen conjugates are unknown. It is possible, as suggested by Pitot and Heidelberger (24), that the binding of the carcinogen to a specific protein capable of acting as a repressor might give rise to a perpetuated change leading to carcinogenesis. In order to answer these questions, it is important to purify these protein-carcinogen conjugates. Tasseron et al. (39) succeeded in extensive purification of the h-protein from mouse skin.

In this communication, we report the purification to molecular size homogeneity of the h-protein from C3H mouse liver. Its physical properties are identical to the mouse skin h-protein. The h-protein from mouse liver is considerably more convenient to purify than the corresponding protein from the mouse skin and is more readily available. We have also isolated other proteins to which the hydrocarbons are bound from the skin and liver.

MATERIALS AND METHODS

Animals. Male C3H mice were obtained through the courtesy of the Drug Development Branch of the National Cancer Institute. The mice were about 12 weeks old at the time of injection. They were fed Rockland pellets and water. Five days before i.p. injection of the carcinogen, phenobarbital sodium (1 mg/ml) was added to their drinking water and was removed 24 hr before injection. This concentration of phenobarbital produces maximum induction of the rat liver microsomal enzymes (20) that activate MCA to bind covalently to the h-protein. Phenobarbital has also been reported to induce ligandin (25, 26).

Chemicals. Tritiated MCA* was obtained from American Cancer Society Professor of Oncology.

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The abbreviations used are: MCA, 3-methylcholanthrene; Buffer 1, 10 mM Tris HCl (pH 8.0) containing 10 mM NaCl and 5 mM β-mercaptoethanol; Buffer 2, 10 mM sodium acetate buffer (pH 5.0) containing 5 mM β-mercaptoethanol; CM-cellulose, carboxymethylcellulose; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; AP-I, acid phosphatase I.

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Purification of the h-protein from Mouse Liver. Eighteen mice were sacrificed by cervical dislocation 48 hr after i.p. injection of 0.1 ml of [3H]MCA solution. The livers were perfused via the hepatic portal vein with ice-cold Buffer 1 and were excised, cut into small pieces, and placed in Buffer 1. All subsequent procedures were carried out at 0–4°C.

The livers were homogenized in an all-glass homogenizer in Buffer 1 and centrifuged at 700 × g for 10 min. The pellet was washed with Buffer 1 and recentrifuged at 700 × g for 10 min. The combined supernatant fractions were centrifuged at 105,000 × g for 1 hr, and the resultant supernatant fraction (the cytosol) was concentrated by ultrafiltration through a Diaflo UM 10 membrane (Amicon Corp., Lexington, Mass.) according to the method of Blatt et al. (5). The subsequent steps involved Sephadex G-25, DEAE-cellulose, and Sephadex G-100 chromatography as described by Tasseron et al. (39). The radioactive peak from the Sephadex G-100 column was concentrated by ultrafiltration, dialyzed overnight against Buffer 2, and then passed through a CM-cellulose column (0.9 x 5 cm) that had been previously equilibrated with Buffer 2. Twenty fractions, 5 ml each, were collected, after which a NaCl gradient (0 to 0.1 M) was initiated. Fractions of 1.5 ml were collected. The radioactive peak containing the h-protein, at about 0.07 M NaCl, was pooled and concentrated by ultrafiltration and dialyzed overnight against Buffer 1. Further concentration to 0.1 ml was achieved by reverse dialysis using Ficoll powder (Pharmacia Laboratories, Inc., Piscataway, N. J.) (39). The concentrate was rechromatographed on a Sephadex G-100 column (2.5 x 100 cm). The radioactive peak containing essentially pure h-protein was pooled and concentrated by ultrafiltration.

Purification of the h-Protein from Mouse Skin. The Sephadex G-100 fraction of the h-protein from mouse skin was treated with [3H]MCA as obtained as described above. The concentrate was rechromatographed on a Sephadex G-100 column (1.5 x 40 cm) and eluted with Buffer 1. Fractions of 2.8 ml were collected, and the radioactive peak containing essentially pure h-protein was pooled and concentrated by ultrafiltration.

Isoelectric Focusing Procedures. Isoelectric focusing in an LKB 8100 electrofocusing column was performed using the method described by Vesterberg and Svensson (40). The pH of the gradient used was either from 3 to 10 or 7 to 10. The gradients were obtained with carrier ampholites (LKB Instruments, Inc., Rockville, Md.). The anode was placed at the top of the column, the cathode at the bottom, and between them a sucrose density gradient containing 1% carrier ampholite. The column was run for 36 hr, and the voltage applied was 300 V, which was increased to 500 V after 12 hr. Fractions of 40 drops were collected. The pH of the fractions was measured with a Radiometer Model 26 pH meter at room temperature. The radioactivity was assayed by taking 0.2 ml of each of the fractions, to which 10 ml of Scintisol was added, and by liquid scintillation counting.

Isoelectric focusing in gels was carried out using the method of Wrigley (41), essentially as reported by Tasseron et al. (39).

Polyacrylamide Gel Electrophoresis. SDS gel electrophoresis was carried out according to the stacking-gel procedure of Laemml (15). Five μg of the protein sample were mixed with 0.25 ml of “sample buffer,” which was made with 10 ml of glycerol, 5 ml of Β-mercaptoethanol, 30 ml of 10% SDS, and 12.5 ml of 0.5 M Tris HCl (pH 6.8) in 0.4% SDS in 100 ml of water. The above protein solution was heated for 2 min at 90°C prior to electrophoresis at 2 mA/gel. For liquid scintillation counting, 2-mm slices of the dried gel were placed in scintillation vials and heated for 1 hr at 60°C in 1 ml of Soluene (Packard Instruments, Inc., Downers Grove, Ill.), and toluene-PPO was added to 10 ml.

Protein Determinations. Unless otherwise specified, the protein concentrations were determined by the method of Lowry et al. (19).

Sedimentation Studies. The sedimentation coefficient of the purified mouse liver h-protein was determined by sedimenting 5 mg/ml in Buffer 1 at 60,000 rpm in a Spinco Model E ultracentrifuge using a double-sector cell. Photographs were taken at 8-min intervals using a double Schlieren optical system. The plot of the log of the refractive index maximum as a function of time was linear, and the S20,w was calculated from the slope (30).

The S20,w of the mouse liver h-protein and the partially purified h-protein from mouse skin were also determined using linear sucrose density gradient centrifugation (5 to 20% in water). Each sample (0.2 ml) was carefully layered at the top of 11 ml of gradient and centrifuged at 180,000 × g for 30 hr. BSA was used as a marker. Fractions of 8 or 16 drops were collected into scintillation vials to which 10 ml of Scintisol was added for determination of the radioactivity. BSA was measured spectrophotometrically using a 2-mm continuous-flow cell in a Model 2400 S Gilford spectrophotometer at 280 nm.

Determination of the Molecular Weight of the h-Protein and the Acidic Protein. The molecular weight of the h-protein was determined by gel filtration using a 2.5-x 100-cm Sephadex G-100 column previously equilibrated with Buffer 1. BSA (Sigma Chemical Co., St. Louis, Mo.); ovalbumin (Worthington Biochemical Corp., Freehold, N. J.); elastase (Worthington), and DNase I from bovine pancreas (Calbiochem, Los Angeles, Calif.) were used as standards.

RESULTS

Incorporation of [3H]MCA into the Basic Protein Fraction of the Mouse Liver Cytosol. The basic proteins do not adhere to DEAE-cellulose when the column is eluted with Buffer 1 (pH 8.0). However, the acidic proteins are retained and are eluted with increasing concentrations of NaCl.
and/or if the pH of the column is lowered. Since the h-protein is relatively basic, the mice were sacrificed after the incorporation of [3H]MCA into the basic fraction reached maximum levels 48 hr after injection, as shown in Chart 1. The levels of MCA-bound proteins decreased after 7 days.

**Purification of the h-Protein from Mouse Liver.** The various steps in the purification of the h-protein from mouse liver are summarized in Table 1. SDS-polyacrylamide gel electrophoresis was used to monitor the stage of purification. The SDS gels of the various steps are presented in Chart 2. Steps 3 through 6 (105,000 x g supernatant fraction; and chromatography on Sephadex G25, DEAE-cellulose, and Sephadex G-100) were carried out as described by Tasseron et al. (39). Although the Sephadex G-100 chromatograms (from Step 6) showed a single symmetrical peak of radioactivity due to the bound [3H]MCA, the SDS gels (Chart 2e) revealed the presence of several protein bands. Therefore, CM-cellulose chromatography (Step 7) was used. The CM-cellulose chromatogram is shown in Chart 3a. The major radioactive peak at about 0.07 M NaCl contained the h-protein (Chart 2f). This procedure is superior to the isoelectric column step used by Tasseron et al. (39), because the h-protein was separated from 2 other basic proteins that have similar molecular weights and to which metabolites of MCA are bound. The CM-cellulose profile of the mouse skin basic proteins (Chart 3b) resembles the corresponding profile of the liver proteins (Chart 3a). Fractions 82 to 96 (Chart 3b) contained the h-protein with a subunit molecular weight of 20,000, which contained all the radioactivity due to bound [3H]MCA. The final step in the purification included a 2nd Sephadex G-100 column (Chart 4). SDS-polyacrylamide gel electrophoresis showed that at this stage of purification the h-protein was more than 95% pure (Chart 2g). All of the radioactivity due to bound [3H]MCA was associated with the major protein band (Chart 2g).

**Properties of the Mouse Liver h-Protein.** The physical properties of the mouse liver h-protein (Table 2) are very similar to the properties of h-protein from mouse skin (39), indicating that these proteins may be identical. The 2 h-proteins resemble ligandin in most physical properties (17) (Table 2). However, the wide range of pl values (8.05 to 9.3) suggests that the h-protein and ligandin are not the same.

The molecular weight of the mouse liver h-protein is 44,000, that is essentially the same as determined by gel filtration through Sephadex G-100. The protein consists of 2 subunits of molecular weight of 20,000, as determined by SDS-polyacrylamide gel electrophoresis, and, as shown in Chart 5, the subunits of the mouse liver protein migrate identically to the subunits of the skin h-protein.

As shown in Chart 6, the isoelectric point of the liver h-protein is 8.05, the same as we previously reported for the skin protein, as determined by column isoelectric focusing (39). The isoelectric point of the liver component was 8.6 when determined by isoelectric-focusing gels. Sedimentation coefficient values of 3.6 S were obtained for the h-proteins from both the mouse liver and skin, both by analytical centrifugation and sucrose density gradient centrifugation.

**Isolation of 2 Minor Basic Proteins to Which [3H]MCA Is Bound.** The radioactive peak from the Sephadex G-100 column (Step 6) contains more than 1 protein to which

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**Table 1**

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity*</th>
<th>% recovery of total radioactivity</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Tissue homogenate</td>
<td>6,250</td>
<td>900</td>
<td>100</td>
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<td>2</td>
<td>700 × g supernatant fraction</td>
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<td>80</td>
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<td>3</td>
<td>105,000 × g supernatant fraction</td>
<td>2,800</td>
<td>850</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>Sephadex G-25</td>
<td>330</td>
<td>4,000</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>DEAE-cellulose</td>
<td>150</td>
<td>4,500</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>Sephadex G-100 (I)</td>
<td>45</td>
<td>10,500</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>CM-cellulose</td>
<td>6</td>
<td>35,000</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Sephadex G-100 (II)</td>
<td>4</td>
<td>38,000</td>
<td>3</td>
</tr>
</tbody>
</table>

* Expressed as dpm/mg protein.

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Chart 1. The incorporation of [3H]MCA into the basic proteins of mouse liver cytosol. Forty-eight mice were given i.p. injections of [3H]MCA as described in "Materials and Methods." Six mice were sacrificed at each time. The liver cytosol fractions were prepared and filtered through a Sephadex G-25 column. The basic protein fractions were obtained from DEAE-cellulose columns (3 x 7 cm) eluted with Buffer 1 (pH 8.0).
Table 2
Comparison of the properties of the mouse liver h-protein, the mouse skin h-protein, and rat liver ligandin (10, 17)

<table>
<thead>
<tr>
<th></th>
<th>$s_{20,w}$</th>
<th>MW x 10^3</th>
<th>Subunit MW x 10^3</th>
<th>pl</th>
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</thead>
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<td>Ligandin</td>
<td>3.5</td>
<td>37–47</td>
<td>23</td>
<td>8.4–9.3</td>
</tr>
<tr>
<td>h-protein (mouse skin)</td>
<td>3.6</td>
<td>40</td>
<td>20</td>
<td>8.0</td>
</tr>
<tr>
<td>h-protein (mouse liver)</td>
<td>3.6</td>
<td>44</td>
<td>20</td>
<td>8.0–8.6</td>
</tr>
</tbody>
</table>

Chart 4. The final Sephadex G-100 chromatogram of the h-protein from mouse liver. The concentrated fraction from the CM-cellulose column (6.0 mg) was dialyzed against Buffer 1, layered at the top of the column (1.5 x 40 cm), and eluted with Buffer 1. Fractions of 2.8 ml were collected.

Chart 5. SDS-polyacrylamide gel profile of total proteins of the Sephadex G-25 fraction from a, mouse liver and b, mouse skin. The gels were sliced into 2-mm fractions, and the radioactivity due to covalently-bound [3H]MCA metabolites was measured as described in "Materials and Methods."
metabolites of [3H]MCA are bound. This was first observed by Tasseron et al. (39) while purifying the h-protein from mouse skin. We accomplished the separation of those labeled proteins by CM-cellulose chromatography. After the elution of the major peak containing the h-protein, a steeper NaCl gradient (0.1 to 0.5 M) was applied (Chart 3). Two basic labeled proteins with a lower affinity to MCA than the h-protein were eluted at about 0.13 and 0.19 M NaCl in both the skin and liver preparations. These proteins, each having a molecular weight of around 45,000, are referred to as "basic protein II" and "basic protein III," respectively. The SDS-polyacrylamide gels of the mouse liver minor proteins are compared with a gel of the h-protein in Chart 7. When the 3 gels were sliced, the radioactive peaks due to the bound MCA were not significantly separated, indicating that they have similar subunit molecular weights of about 20,000. The basic proteins II and III recovered from the CM-cellulose column constituted about 1.5% of the soluble proteins of the 105,000 x g supernatant fraction (11% of the soluble proteins of the Sephadex G-25 fraction). Only 2% of the total radioactivity was associated with these proteins. On the other hand, the h-protein recovered from the same CM-cellulose column constituted only 0.2% of the soluble proteins of the cytosol (1.8% of the proteins of the Sephadex G-25 fraction). However, the radioactivity associated with the h-protein was 4% of the total radioactivity. These results are summarized in Table 3.

Isolation of Acidic Proteins to Which [3H]MCA Is Bound. After the basic proteins from mouse liver and skin cytosols were eluted from DEAE-cellulose columns using Buffer I, the pH of the columns was lowered to 5.0 with 250 ml of Buffer 2 containing 0.1 M NaCl. Six-ml fractions were collected. The columns were then successively eluted with two 250-ml volumes of Buffer 2 containing 0.25 and 0.5 M NaCl (Chart 8).

The acidic protein from mouse liver cytosol that was eluted with 0.1 M NaCl, termed AP-I, was partially characterized and subjected to further purification. The radioactive fraction from the DEAE-cellulose column con-
A radioactive protein was obtained at 0.18 M NaCl, which was concentrated by ultrafiltration and dialyzed against Buffer 1. SDS gels of this fraction indicate that the acidic protein is not homogeneous and requires further purification (more than 10 bands were observed). The AP-I has a molecular weight of 60,000, as determined by gel filtration using a Sephadex G-100 column (2.5 x 100 cm). A pI value of 5.0 was obtained with an isoelectric focusing column (Chart 11). The protein has a sedimentation coeffi-
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cient of 4.5 S, as measured on sucrose density gradients.

The other acidic proteins that were eluted from the DEAE-cellulose columns (Chart 8) were found to consist of several labeled proteins when subjected to Sephadex G-100 filtration.

**Mode of Binding of MCA and/or Metabolites to the h-Protein and to the Acidic Protein in Vivo.** Metabolites of MCA are covalently bound to the h-protein. This has been concluded from several experiments with the purified protein from the liver: (a) upon denaturation by SDS-gel electrophoresis, the h-protein band retained the radioactivity of bound [3H]MCA (Chart 5); (b) the radioactivity from the h-protein preparation was essentially nonextractable into chloroform; and (c) when the [3H]MCA-labeled h-protein (15,000 dpm) was treated with 10% trichloroacetic acid, most of the radioactivity remained with the denatured protein after filtration on glass fiber discs. Only 3000 dpm were recovered in the filtrate. Similarly, [3H]MCA metabolites were found to be covalently bound to the 2 minor basic proteins.

On the other hand, the binding of [3H]MCA to the mouse liver AP-I appears to be noncovalent, based on the following evidence: (a) upon denaturation and SDS-gel electrophoresis, the radioactivity of bound [3H]MCA was lost because of the dissociation of the carcinogen-protein complex; (b) most of the radioactivity of the [3H]MCA AP-I complex (2200 dpm) was extractable into chloroform (1800 dpm); and (c) when the complex (7500 dpm) was denatured with 10% trichloroacetic acid, no radioactivity was associated with the denatured protein. About 7000 dpm were recovered in the filtrate.

**DISCUSSION**

In this communication we report the isolation from mouse liver and skin cytosols of 3 basic proteins and 1 acidic protein to which MCA and its activated metabolites are bound. The major basic protein that has the highest affinity for the carcinogen metabolites has been purified from the liver. The purification procedure involves successive fractionations on Sephadex G-25, DEAE-cellulose, and Sephadex G-100 using the procedure of Tasseron et al. (39) for partial purification of the principal MCA-binding protein from mouse skin (the h-protein). Following the Sephadex G-100 step, the protein is subjected to CM-cellulose chromatography, and they constitute about 2% of the total soluble proteins. It now seems probable that these proteins, rather than the h-protein, are identical to ligandin (B. Ketterer, A. Sarrif, and C. Heidelberger, unpublished observations). To investigate these possibilities we are now in the process of developing anti-h-protein serum and will test antiligandin serum against the h-protein, basic proteins II and III, and ligandin. The mouse liver h-protein is not similar in physical properties to the principal azo dye h$_{r}$-5S protein (34–36).

Our interest in the h-protein stems from the previous finding of Abell and Heidelberger (1) of the close correlation between the extent of binding of the hydrocarbon to this mouse skin protein and the carcinogenic activity of the compound. Abell and Heidelberger (1) and Sorof et al. (37) demonstrated that, in almost all cases, the protein-carcinogen conjugate is undetectable in tumors produced by hydrocarbons and azo dyes, respectively. Baldwin et al. (3) and Bannikov et al. (4) showed immunologically that ligandin is deleted in azo dye-transformed tumors. Most recently, Mott et al. (23) found that the level of the principal slow h$_{r}$-5S azo-protein is reduced in primary tumors induced by azo dye carcinogens but not in transplanted, differentiated tumors induced by other carcinogens, which might have their own target proteins.

A relatively acidic protein to which MCA is bound noncovalently has been partially purified and characterized from the mouse livers. A similar protein has also been identified in the skin. We have termed this AP-I, which has a molecular weight of 60,000, similar to that of mouse serum albumin. The protein may also be similar to the protein described by Slaga et al. (32), to which carcinogenic hydrocarbons bind specifically in vitro. A protein that somewhat resembles AP-I has been partially purified from rat livers by Filler et al. (7). It has a molecular weight of 67,000 and a pI of 6.7 (AP-I has a molecular
weight of 60,000 and a pI of 5.0). This protein appears to be different from the corticoid hormone receptor (corticoid binder II) \(^{(7, 31)}\).

These studies show that there are several proteins to which MCA and metabolites are bound, the major one being the \(h\)-protein. Whether any of these proteins individually or collectively play a role in the process of carcinogenesis is yet to be determined. They may prevent the carcinogen from leaking from the cell, thus increasing the probability that the carcinogen will interact with its target sites. On the other hand, they may partially protect the cell by trapping the carcinogen and facilitating its excretion.

One protein might transport the carcinogen to the nucleus where the carcinogen could be released to specific sites in the chromatin, leading to cell transformation, as was postulated in 1962 by Abell and Heidelberger \(^{(1)}\). It is also possible that the carcinogen-protein conjugate may act as an abnormal derepressor, leading to a perpetuated change, as proposed by Pitot and Heidelberger \(^{(24)}\).

The recent developments in the role of hormone receptors in enzyme induction and cell differentiation may serve as the basis for analogous studies to investigate any role the various carcinogen-protein conjugates may play in the process of carcinogenesis. In this laboratory, a cell line derived from C3H mouse embryo has been developed recently \((27, 28)\). This cell line, which is highly sensitive to postconfluence inhibition of cell division and which has been shown to undergo malignant transformation in response to carcinogenic hydrocarbons \((27, 28)\), provides the material to test the possible role of the \(h\)-protein and the other hydrocarbon protein complexes in the process of chemical carcinogenesis.

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