Rat Hepatoma Reuber H-35 Cells Produce a 2-Cell Stage-Specific Inhibitor of the Cleavage of Mouse Embryos

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ABSTRACT

We purified an embryonic stage-specific inhibitor produced by rat hepatoma Reuber H-35 cells against cleaving mouse 2-cell embryos and defined its biological properties.

Zygotes obtained from CD-1 mice (a strain that shows a 2-cell block in vitro) or C57BL/6 and B6C3F, mice (strains that do not) were cultured in media with and without 50 μM EDTA, respectively. The development of the zygotes from all strains was arrested at the 2-cell stage when zygotes were cocultured with Reuber H-35 cells. However, the embryos from C57BL/6 and B6C3F, were less sensitive than those from CD-1 against the inhibitory effects of development. This inhibitory effect was also evident in medium conditioned with the Reuber H-35 cells. The factor from the conditioned medium was separated into its < 10 000 Mr fraction by ultrafiltration and was further purified in fraction B-25 as a single peak by reverse-phase column chromatography. An incubation as short as 3-h during the late 2-cell stage (G2 phase) with fraction B-25 suppressed cleavage in 61.5% of the CD-1 embryos (30.3% in control culture). Although the inhibitory effect was reversible, embryos that cleaved again either degenerated or were retarded at various stages in their subsequent development. Additionally, a long-term incubation of developing zygotes with the inhibitory factor caused a significant reduction in [3H]thymidine (TdR) incorporation into the DNA of CD-1 2-cell embryos as well as developmental arrest at the interphase of the 2-cell stage.

These results indicated that this factor will serve as a valuable tool with which to clarify the proliferating mechanism of the preimplantation embryo.

INTRODUCTION

A number of nutritional and environmental factors regulate the preimplantation development of mammalian embryos. In all species studied, developmental arrest occurred at specific cell stages in cultured embryos including mouse 2-cell [1, 2], human 4- to 8-cell [3], hamster 2-cell [4], sheep and goat 8- to 16-cell [5], cow 4- to 8-cell [6], and pig 4-cell [7] embryos.

Developmental arrest has been studied most extensively in mice [1, 2]. Most of the zygotes obtained from outbred strains, and some from inbred strains, do not develop to the blastocyst but arrest at the 2-cell stage when cultured in chemically defined medium [1, 2]. This phenomenon is referred to as the “2-cell block” [1, 2]. Various procedures to release embryos from the 2-cell block have been described by several investigators; these include improvements in defined culture media, such as modification of inorganic constituents [8–11] and energy sources [11] and addition of amino acids [9, 11], to support high rates of zygote development in blocking strains. Recently it was also reported that adding the free radical scavenger superoxide dismutase [12, 13] or the growth factor activin A [13, 14] to the medium positively affected development. Muggleton-Harris and her colleagues [15, 16] reported that the block could be overcome by transplanting the cytoplasm from a nonblocked embryo into a blocked embryo, and they demonstrated cell-cycle-related cytoplasmic components. However, the molecular mechanism or mechanisms underlying the 2-cell block in mice have not yet been fully defined.

The liver synthesizes and secretes some of the serum components, and serum fractions affect the early development of mouse embryos [17]. However, the exact substrates synthesized by hepatocytes, and their functions, remain unclear. In this study, we purified and characterized the biological properties of a unique factor synthesized by rat hepatoma Reuber H-35 cells grown in a chemically defined medium. This factor affected the early development of mouse embryos.

MATERIALS AND METHODS

Media

M2 [18], modified Krebs-Ringer solution (TYH) [19], and modified Whitten’s medium (WM) [20] were supplemented
with 3, 4, and 3 mg/ml BSA (fraction V; Intergen, Purchase, NY), respectively. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). All media were prepared with freshly distilled type I (18 megaohm) water and were not supplemented with phenol red.

Embryo Collection

To obtain in vivo-fertilized embryos, 6- to 12-wk-old virgin female mice of the CD-1 (random bred, Swiss; Japan Crea, Tokyo, Japan), C57BL/6 (inbred; Sankyo Labo Service, Tokyo, Japan), and B6C3F1 (an F1 hybrid of two inbred strains, C57BL/6 × C3H/He; Sankyo Labo Service) strains were housed under controlled illumination (lights-on from 0800 to 2000 h) at 22°C. The females were induced to superovulate by i.p. injections of 5 IU eCG (Teikoku Zohki, Tokyo, Japan) followed 48 h later with 5 IU hCG (Sankyo), and they were then mated overnight with fertile males (15- to 30-wk-old) within each strain. One-cell embryos were flushed in M2 from excised oviducts 21–22 h after hCG injection. Cumulus cells were removed from fertilized ova with hyaluronidase (150 U/ml in TYH without BSA, type I-S; Sigma Chemical Company, St. Louis, MO).

For in vitro-fertilized embryos, all incubations proceeded under a small amount of mineral oil (Squibb & Sons, Princeton, NJ) in Falcon tissue culture dishes (1008, Falcon; Fisher Scientific, Boston, MA) at 37°C with 5% CO₂ in air. Spermatozoa were collected from the caudal epididymis of fertile CD-1 (12- to 30-wk-old) males and incubated in 0.4 ml TYH for 1 h before insemination. Cumulus-enclosed oocytes were recovered from the oviducts of CD-1 female mice at 16.5 h after hCG and transferred to TYH. Insemination proceeded with an appropriate volume of an incubated epididymal spermatozoa suspension to give a final spermatozoa concentration of 1 × 10⁶ cells per milliliter. After a 5-h incubation, embryos with one or two polar bodies and no signs of degeneration were selected for further culture and washed twice with WM.

Culture of Hepatoma Cells and Embryos

Rat hepatoma Reuber H-35 cells were provided by Dr. A. Niwa (Dokkyo University School of Medicine, Tochigi, Japan). Human hepatoma Hep G2 cells were obtained from the ATCC (Rockville, MD). The cells were routinely maintained in DMEM supplemented with 2% and 10%, respectively, heat-inactivated fetal calf serum (Filtron, Altona, Australia) at 37°C in 5% CO₂ in air. Culture without serum proceeded as previously described [21, 22]. Briefly, the cell lines were cultured for 2 days in serum-free DMEM containing 5 μg/ml human transferrin, 5 ng/ml selenious acid, and 20 μM ethanolamine (DMEM-S). The medium was changed daily. Reuber H-35 or Hep G2 cells (2–5 × 10⁵ cells per well in 4-well dishes; Nunc, Roskilde, Denmark) were washed twice with 1 ml Dulbecco's PBS (Nissui Pharmaceutical); then 10–30 zygotes randomly assigned in each group were transferred to the wells and cultured in 1 ml WM with or without 50 μM EDTA at 37°C in 5% CO₂ in air. Wells for the control culture were similarly processed in the absence of hepatoma cells. Unless otherwise indicated, CD-1 zygotes were cultured with medium in the presence of 50 μM EDTA. Distilled water was placed in the outer well space for humidification. Embryonic development was examined at 24-h intervals until 137 h after hCG injection. The viability of the hepatoma cells was assessed by staining with 0.15% trypan blue.

Preparation, Ultrafiltration, and Column Chromatography of Medium Conditioned with Reuber H-35 Cells

Confluent Reuber H-35 cells in 80-cm² dishes, cultured with DMEM-S for 2 days, were washed twice with 15 ml Dulbecco's PBS and further incubated with 20 ml of WM without BSA for 24 or 48 h. The cell-conditioned medium was immediately cooled to 4°C, centrifuged at 1500 × g for 15 min to remove cell debris, and passed through a 0.20-μm filter (DISMIC-25; Advantec, Tokyo, Japan) and separated into two fractions according to the manufacturer's instructions (fraction retained on the filter, Mₚ > 10 000; pass-through fraction, Mₚ < 10 000). After addition of BSA (3 mg/ml), these fractions were frozen at −80°C until use for cell culture.

The pass-through fraction (Mₚ < 10 000) without BSA was further applied to a reverse-phase column (ProRPC HR 5/10, 5 × 100 mm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.1% trifluoroacetic acid, and then eluted with a linear gradient of 2-propanol/acetonitrile (1:1 [v/v]) containing 0.1% trifluoroacetic acid at room temperature for 45 min. The flow rate was 0.7 ml/min. The fractions were lyophilized to remove trifluoroacetic acid, 2-propanol, and acetonitrile and then reconstituted with WM to their original volumes.

[^3H]Thymidine (TdR) Incorporation into the DNA of Embryos

DNA synthesis by the embryos was assessed by [³H]TdR incorporation and autoradiography. Zygotes fertilized in vivo were cultured in WM supplemented with or without the Mₚ < 10 000 fraction or 30 μM hypoxanthine in the presence of 50 μM EDTA from 17 h after hCG injection. Immediately after cleavage to 2 cells, the embryos were cultured in the same media containing 2 μCi/ml [³H]TdR. Sixty-four hours after hCG injection, 7–8 embryos were harvested into a tube, fixed for 30 min in 5% trichloroacetic acid (TCA) at 4°C, and washed three times with 5% TCA at 4°C. The
FIG. 1. The effect of coculture with hepatoma cells on the in vitro development of zygotes obtained from the blocking strain. CD-1 zygotes fertilized in vivo were co-cultured with rat hepatoma Reuber H-35 (+EDTA + Reuber H-35) or human hepatoma Hep G2 cells (+EDTA + Hep G2) (2-5 × 10^5 cells per well) in the presence of 50 μM EDTA. Other groups were cultured without EDTA and hepatoma cells (-EDTA), or with 50 μM EDTA alone (+EDTA). The percentages of embryos in groups + EDTA + Reuber H-35 and + EDTA + Hep G2 that reached normal developmental parameters (41 h after hCG, 2-cell stage; 65 h after hCG, 4-8-cell stage; 89 h after hCG, morula-blastocyst stage) were compared with that of embryos in the + EDTA group. The number of zygotes cultured is indicated in each column. At least six separate experiments per group were completed. **p < 0.01; ***p < 0.001.

amount of radioactivity of [3 H]TdR incorporated into the DNA was determined in a liquid scintillation counter. Parallel cultures were processed for autoradiographic analysis. The embryos cultured with [3 H]TdR were placed on slides and fixed. The slides were dipped into a Konica NR-M2 emulsion (Konica, Tokyo, Japan) and exposed at 4°C for 1 wk. Autoradiograms were developed in Kodak GBX (Eastman Kodak, Rochester, NY), and chromosomes were stained with Giemsa solution.

Statistical Analysis

The data were compared according to the percentage of embryos that reached normal development and were analyzed by chi-square test. The statistical significance of differences between sample means was determined by Student's t-test. Data are expressed as the mean ± SE.


<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Incubation period after hCG (h)*</th>
<th>No. of zygotes culturedb</th>
<th>No. (%) of embryos 65 h after hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24-30</td>
<td>20</td>
<td>0 (%)</td>
</tr>
<tr>
<td>1-Cell</td>
<td>24-36</td>
<td>20</td>
<td>0 (%)</td>
</tr>
<tr>
<td>Early 2-cell</td>
<td>24-42</td>
<td>20</td>
<td>0 (%)</td>
</tr>
<tr>
<td>Mid 2-cell</td>
<td>24-48</td>
<td>20</td>
<td>1 (%)</td>
</tr>
<tr>
<td>Late 2-cell</td>
<td>24-54</td>
<td>20</td>
<td>0 (%)</td>
</tr>
<tr>
<td>Late 2-cell</td>
<td>24-54</td>
<td>20</td>
<td>1 (%)</td>
</tr>
</tbody>
</table>

*CD-1 zygotes fertilized in vivo were cocultured with Reuber H-35 cells in the presence of 50 μM EDTA for the indicated periods. The embryos were cultured in fresh 50 μM EDTA (from hCG injection (from

Inhibition of Cleavage of Mouse 2-Cell Embryos by Reuber H-35 Cells or Cell-Conditioned Medium

The effects of coculture with hepatoma cells on the in vitro development of CD-1 zygotes (blocking strain) are summarized in Figure 1. More than 90% of the Reuber H-35 cells were alive on the third day of culture with WM, whereas more than 50% of the Hep G2 cells were dead on the second day of culture as assessed by trypan blue staining. As there was no effect attributable to cell density between 2 and 5 × 10^5 cells per well, data from the same procedures were combined for analysis.

In the presence of 50 μM EDTA in WM, more than 70% of the zygotes had developed to the 4-8-cell stage at 65 h after hCG, whereas about half reached the 4-8-cell stage in the absence of EDTA. When zygotes were cocultured with Reuber H-35 cells in medium containing 50 μM EDTA, development was potently inhibited at the 2-cell stage in all embryos; less than 0.5% of them reached the morula-blastocyst stage even after 89 h. A similar phenomenon was observed when zygotes were cultured in WM conditioned with Reuber H-35 cells for 24 or 48 h (data not shown). The inhibitory effect of the WM conditioned for 48 h was weaker than that of the WM conditioned for 24 h (data not shown). However, the subsequent development of 4- and 8-cell embryos obtained from CD-1 mice was inhibited by coculture with Reuber H-35 cells or by incubation in the conditioned medium (data not shown). On the other hand, the development of zygotes was partially inhibited at the 4-8-cell stage by coculture with human hepatoma Hep G2 cells, but more than 60% of the embryos reached the morula-blastocyst stage.

As shown in Table 1, we examined the time-dependent effect of cocultivation with hepatoma cells on the development of embryos. CD-1 zygotes fertilized in vivo were cultured with Reuber H-35 cells in the presence of 50 μM EDTA for various periods; then the embryos were washed and transferred to fresh WM with 50 μM EDTA. More than 18 h of coculture from 24 h after the hCG injection (from
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FIG. 3. Elution profile of the $M_r < 10\,000$ fraction by reverse-phase column chromatography. Medium conditioned with Reuber H-35 cells was ultrafiltrated. The pass-through fraction ($M_r < 10\,000$) was further separated on a reverse-phase column equilibrated with 0.1% trifluoroacetic acid. As shown by the broken line, the column was eluted with a linear gradient of 2-propanol/acetonitrile (1:1 [v/v]) containing 0.1% trifluoroacetic acid as described in Materials and Methods. Hypoxanthine and adenosine were eluted in the flow-through fraction (fractions 6–12 and 13–18 in fraction $A$, respectively).

FIG. 2. The effect of coculture with Reuber H-35 cells on the in vitro development of zygotes obtained from nonblocking strains. In vivo-fertilized zygotes from C57BL/6 or B6C3F1 were cultured with (Reuber H-35) or without (Control) Reuber H-35 cells in the absence of 50 μM EDTA. The percentages of embryos reaching normal developmental parameters (41 h after hCG, 2-cell stage; 65 h after hCG, 4–8-cell stage; 89 h after hCG, morula-blastocyst stage) were compared. The number of zygotes cultured is indicated in each column. Four separate trials were conducted. *$p < 0.05$; **$p < 0.001$.

Separation of the Factor That Inhibited Developing 2-Cell Embryos in the WM Conditioned with Reuber H-35 Cells

The substrates in the Reuber H-35 cell-conditioned WM were separated into two fractions to characterize the molecular properties of the inhibitory factor against the developing 2-cell embryos. Zygotes obtained from CD-1 mice were cultured with these fractions in the presence of 50 μM EDTA. Development at the 2-cell stage was arrested by the original conditioned WM (92% of zygotes cultured) and the $M_r < 10\,000$ fraction (80% of the zygotes cultured). In contrast, the $M_r > 10\,000$ fraction exerted no effect. The $M_r < 10\,000$ fraction slightly inhibited the development of zygotes from C57BL/6; only 30% of those cultured were arrested at the 2-cell stage.

The $M_r < 10\,000$ fraction was further separated by reverse-phase column chromatography as shown in Figure 3. So that a clear effect of these fractions could be seen, the effects of the eluted fractions on the developing 2-cell embryos from CD-1 mice were examined; these are summarized in Table 2. Development at the 2–3-cell stage was arrested in zygotes cultured with fraction B (62.9% of zygotes cultured). Although fraction B-28, a major subpeak fraction of fraction B, had no effect on the development of zygotes, fraction B-25 markedly inhibited development at...
The Most Effective Target Stage of the Factor during the 2-Cell Stage

We examined the stage-specific and short-term effect of fraction B-25 in more detail. CD-1 zygotes fertilized in vitro were incubated with the fraction during various 2-cell stages; then the embryos were washed and transferred to fresh WM. As shown in Table 3, the cleavage of the 2-cell embryos was potently inhibited in groups incubated with the fraction during the late 2-cell stage, particularly between 29 and 32 h after in vitro fertilization. About 62% of the cultured zygotes were arrested at the 2–3-cell stage, and the duration of the 2-cell stage was extended ~6 h by an incubation with fraction B-25. More than 95% of the arrested embryos cleaved again, and about 60% of these degenerated or arrested again later (data not shown).

Similar results were obtained after an incubation with the Mᵣ < 10,000 fraction (data not shown), suggesting that fraction B-25 was the main contributor to the inhibitory effect of the Mᵣ < 10,000 fraction.

The Effect of the Inhibitory Factor on Incorporation of \(^{3}H\)TdR into the DNA of 2-Cell Embryos

The effect of a long-term incubation with inhibitory factor on DNA synthesis of 2-cell embryos was assessed by incorporation of \(^{3}H\)TdR into the DNA. As shown in Table 4, \(^{3}H\)TdR incorporation was significantly inhibited (p < 0.05) to 21.5% of that in the control culture by incubation with the Mᵣ < 10,000 fraction until 64 h after hCG; on the other hand, the incorporation was partially, but not significantly, suppressed by 30 μM hypoxanthine (43.1% of control culture). Autoradiographic analysis confirmed that the \(^{3}H\)TdR was incorporated into the nuclei of 2-cell embryos. The silver grains and chromosomal patterns revealed that the blastomeres had nuclei with decondensed, interphase DNA (data not shown).

### Table 2. The effects of various subfractions of the Mᵣ < 10,000 fraction separated by reverse-phase column chromatography upon the in vitro development of CD-1 mouse zygotes.

<table>
<thead>
<tr>
<th>Fractions elutedᵃ</th>
<th>No. of trials</th>
<th>No. of zygotes cultured</th>
<th>2-3-cell</th>
<th>4-cell</th>
<th>degenerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>56</td>
<td>14 (25.0)</td>
<td>42 (75.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mᵣ &lt; 10,000</td>
<td>3</td>
<td>39</td>
<td>24 (61.5)***</td>
<td>15 (38.5)***</td>
<td>0 (0)</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>20</td>
<td>6 (30.0)</td>
<td>14 (70.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>35</td>
<td>22 (62.9)***</td>
<td>10 (28.6)***</td>
<td>3 (8.6)</td>
</tr>
<tr>
<td>B-25</td>
<td>3</td>
<td>29</td>
<td>22 (75.9)***</td>
<td>7 (24.1)***</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B-28</td>
<td>3</td>
<td>29</td>
<td>4 (13.8)</td>
<td>25 (86.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>13</td>
<td>3 (23.1)</td>
<td>9 (69.2)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>13</td>
<td>2 (23.1)</td>
<td>9 (69.2)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>13</td>
<td>4 (30.8)</td>
<td>8 (61.5)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>37</td>
<td>2 (5.6)</td>
<td>34 (91.9)</td>
<td>1 (2.7)</td>
</tr>
</tbody>
</table>

ᵃThe Mᵣ < 10,000 fraction was separated by reverse-phase column chromatography as shown in Figure 3. CD-1 zygotes fertilized in vivo were cultured with WM containing various eluted materials in the presence of 50 μM EDTA. Control embryos (Control) were cultured in WM with 50 μM EDTA alone.

***Significantly different from the control value within each column (p < 0.001).

### Table 3. The effect of incubation with fraction B-25 during various cell stages on the development of CD-1 mouse zygotes.

<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Incubation period after IVF (h)ᵇ</th>
<th>No. of zygotes culturedᶜ</th>
<th>No. (%) of embryos 47 h after IVF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-cell</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>76</td>
<td>6 (7.9)</td>
</tr>
<tr>
<td>1- to Early 2-cell</td>
<td>6-20</td>
<td>73</td>
<td>6 (8.2)</td>
</tr>
<tr>
<td>Early 2- to mid 2-cell</td>
<td>20-23</td>
<td>71</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mid 2-cell</td>
<td>23-26</td>
<td>70</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mid 2- to late 2-cell</td>
<td>26-29</td>
<td>68</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Late 2-cell</td>
<td>29-32</td>
<td>65</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Late 2-cell</td>
<td>32-35</td>
<td>73</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1-Cell</td>
<td>6-47</td>
<td>74</td>
<td>8 (10.8)</td>
</tr>
</tbody>
</table>

ᵇCD-1 zygotes fertilized in vitro (IVF) were incubated with fraction B-25 in the presence of 50 μM EDTA during the various cell stages. The embryos were washed, transferred, and further cultured in fresh WM with 50 μM EDTA. Control embryos (Control) were cultured in WM with 50 μM EDTA alone.

ᶜTwo separate trials were conducted.

*Significantly different from the control value within each column (p < 0.05).

***Significantly different from the control value within each column (p < 0.001).
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In this report we describe the effect of an inhibitor derived from rat hepatoma Reuber H-35 cells upon the cleavage of 2-cell mouse embryos. Since the factor was eluted in fraction B-25 by reverse-phase column chromatography, we refer to it as "Fr. B-25."

The biological and molecular properties of Fr. B-25 can be summarized as follows. As Fr. B-25 exerted no effect on the subsequent development of 4- and 8-cell embryos, it stage-specifically inhibited the development of mouse embryos from blocking and nonblocking strains at the 2-cell stage. Fr. B-25 contains substances with a relatively low molecular weight ($M < 10,000$) that are probably unstable, since the inhibitory activity of WM conditioned for 48 h with Reuber H-35 cells was weaker than that of the WM conditioned for 24 h.

The inhibitory effects of Fr. B-25 on the development of mouse 2-cell embryos closely resemble the biological properties of purines [23–25]. Fissore et al. [24] have reported that developing 2-cell embryos are arrested at interphase by hypoxanthine. Under our conditions, 30 μM hypoxanthine also suppressed the development of CD-1 embryos at the 2-cell stage (72% of the zygotes cultured; authors' unpublished results). However, some differences are evident. Loutradis et al. [25] reported that hypoxanthine did not affect the development of late 2-cell CD-1 embryos and that a hypoxanthine block was partially suppressed by addition of EDTA to the medium. However, the Fr. B-25 block was not suppressed as found in CD-1 embryos. Moreover, Fr. B-25 inhibited $[^{3}H]Tdr$ incorporation into the DNA of 2-cell embryos, whereas hypoxanthine did not significantly suppress the incorporation as indicated here and in another study [23]. The elution profiles of hypoxanthine and Fr. B-25 from reverse-phase column chromatography were different. These data suggest that Fr. B-25 is not hypoxanthine.

It is necessary to define whether Fr. B-25-like substances are secreted only by liver-derived cell lines or also by other cell lines and primary cultures. Until recently, the effect of coculture with liver-derived cells on the development of mouse embryos had not been extensively studied. The present study showed that via coculture with human hepatoma Hep G2 cells, the development of zygotes obtained from CD-1 mice was partially inhibited at the 4–8-cell stage. We considered the possibility that this developmental arrest was attributable to inappropriate culture conditions mediated by the death of Hep G2 cells. Voelkel and Hu [26] have reported that buffalo rat liver (BRL) cells allowed the coculture of bovine zygotes. They noted [26] that the BRL cells supported development of mouse zygotes through the 2-cell block. Considering that established cell lines derived from hepatocytes have lost some differentiated functions, such as the ability to produce plasma proteins [27], Hep G2 and BRL cells may synthesize little Fr. B-25. However, the liver is a major site of the synthesis and secretion of serum components. Ogawa et al. [17] reported that the $M < 1000$ fraction of human cord serum separated by ultrafiltration significantly reduced embryonic development in B6C3F1 mice, particularly at the 2-cell stage. This observation suggests that human cord serum contains hypoxanthine or Fr. B-25-like substances.

On the other hand, many researchers have described the coculture of mouse embryos with other somatic cells in primary cultures, such as uterine cells [28], oviduct cells [28, 29], and dissected ampullae [30]. Few investigators have described the induction of developmental arrest. Lai et al. [31] have demonstrated developmental arrest of CB6F1 mouse embryos at the 6-cell stage by coculture with monkey kidney-derived Vero cells. Our study is the first report of a factor derived from hepatoma cells that potently suppresses the cleavage of 2-cell embryos.

The mechanism(s) underlying Fr. B-25 action with respect to the inhibition of cleavage of 2-cell embryos remains unknown. In our study, the developmental arrest at interphase of the 2-cell stage induced by long-term incubation with Fr. B-25 was reversible. However, the embryos that underwent arrest cleaved again, degenerated, or underwent arrest again later. Concurrently, $[^{3}H]Tdr$ incorporation into the DNA of 2-cell embryos was inhibited. In addition, the most effective target stage for Fr. B-25 is the late 2-cell (G2 phase). Progression through the cell cycle is marked by a series of irreversible transitions that separate the discrete tasks necessary for cell duplication [32, 33], and these transitions are negatively regulated by signals that constrain the cell cycle until specific conditions are fulfilled. Entry into mitosis, for example, is inhibited by incompletely replicated DNA or DNA damage [34, 35]. These findings suggest that incomplete chromosomal replication in the 2-cell embryos incubated with Fr. B-25 causes developmental arrest or cell death. It is also widely accepted that the 2-cell stage is especially important as specific developmental transitions coincide, including the activation of transcription by the embryonic genome (during the mid-2-cell stage) [2, 36] and the selective inactivation or destruction of much of the preexisting maternal mRNAs (during the late 2-cell stage) [2, 37]. We consider that the factor affects two steps in the cell cycle progression.

### TABLE 4. The effect of the $M < 10,000$ fraction or hypoxanthine on the incorporation of $[^{3}H]$thymidine into the DNA of CD-1 2-cell embryos.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>No. of zygotes cultured</th>
<th>$[^{3}H]$Thymidine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>26.0 ± 5.0 (100)</td>
</tr>
<tr>
<td>$M &lt; 10,000$</td>
<td>21</td>
<td>5.6 ± 0.8* (21.5)</td>
</tr>
<tr>
<td>30 μM hypoxanthine</td>
<td>21</td>
<td>11.2 ± 1.0 (43.1)</td>
</tr>
</tbody>
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*CD-1 zygotes fertilized in vivo were cultured with the $M < 10,000$ fraction or 30 μM hypoxanthine in the presence of 50 μM EDTA. Control embryos (Control) were cultured in WM with 50 μM EDTA alone. Immediately after cleavage to 2 cells, the embryos were selected and cultured in the same medium containing 2 μCi/ml $[^{3}H]$thymidine.

*om/embryo, mean ± SE.

*Significantly different from the control value (p < 0.05).
of 2-cell embryos. It may suppress the onset or progression of S and G2 phases indirectly and directly, respectively.

In conclusion, we demonstrated that Fr. B-25 derived from rat hepatoma Reuber H-35 cells is an embryonic stage-specific inhibitor of the cleavage of mouse 2-cell embryos. We are currently clarifying the essential molecule in Fr. B-25 and the molecular mechanism of its action in the preimplantation development of mouse embryos.

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