A limited upstream region of the human sucrase–isomaltase gene confers glucose-regulated expression on a heterologous gene

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We have previously shown that glucose can exert a repressive effect on the transcription of the sucrase–isomaltase (SI) gene in the differentiated enterocyte-like human colon carcinoma cell lines HT-29 and Caco-2. To characterize the region through which glucose exerts this effect, three different-length fragments of the 5′-flanking region of the human SI gene were linked to the reporter gene luciferase in an episomal vector carrying a hygromycin resistance gene. These fragments were used for trans-

fection into a clone of the Caco-2 cell line, PF11, which has high glucose consumption and only expresses SI at high levels when cultured in the presence of a low supply of glucose. By using the stably transformed PF11 cells grown either in standard high glucose (25 mM) or in low glucose (1 mM) it was possible to show that the smallest fragment of the SI promoter, extending from bases –370 to +30, contains all the information required for the glucose repression of the reporter gene luciferase.

INTRODUCTION

Sucrase–isomaltase (SI) is an intestine-specific disaccharidase: in adults, sucrase and isomaltase activities are detected exclusively in the brush border of the small intestine [1,2]. A single gene encodes this enzyme, and the complete SI cDNA and upstream region of the gene in humans have been cloned and sequenced [3,4]. During the past three years most studies on the regulation of the SI gene have been directed towards understanding the molecular mechanisms that account for the tissue-specific expression and for the change in expression that occurs along the crypt–villus axis during enterocytic differentiation [4–6]. Various nuclear protein binding sites have been characterized in the promoter region of the SI gene and one of these is thought to play a role in tissue-specific expression [7,8].

As yet the molecular bases for the changes in expression of SI during development or in carcinogenesis have not been elucidated. In humans the SI gene is expressed at high levels in the fetal colon between the 12th and the 25th week of gestation [9–11] and, although only poorly expressed in normal adult colon [12], is up-regulated in some colon cancers [10,13–15]. Interestingly, in both these situations there is an accumulation of intracellular glycogen [16], this being a reflection of the state of the glucose metabolism of the cells. A potential role for glucose in the regulation of the SI gene was also indicated by results obtained with two enterocyte-like differentiating colonic adenocarcinoma cell lines, HT-29 and Caco-2 [17–19]. We showed that SI expression is low in all culture conditions that lead to an increase in glucose utilization, e.g. in Caco-2 cells treated with forskolin [20,21] or monensin [22,23] and in glucose-deprived HT-29 cells switched back to a medium containing 25 mM glucose [18]. In each case this regulation occurred at the level of

transcription [3,24]. A corroboration of the repressive effect of glucose on SI gene transcription came from results obtained with clones isolated from the Caco-2 cell line. Although they all share the same morphological differentiation and the same pattern of post-confluent increase of sucrase activity and decrease of the rate of glucose consumption in standard culture conditions (25 mM glucose) [25], they differ greatly in their level of glucose utilization and SI expression. Among them, the PF11 clone shows the higher glucose consumption and the lowest SI mRNA and sucrase activity levels [25]. Furthermore, culturing PF11 cells in the presence of 1 mM instead of 25 mM glucose resulted in a 10-fold increase in SI mRNA and activity levels without any effect on cell growth or differentiation [25].

Our aim has been to characterize the region through which glucose exerts its repressive effect on SI gene transcription. Here we describe the transfection of PF11 cells with vectors containing a reporter gene under the control of different upstream regions of the SI gene. As the onset of SI gene transcription occurs after confluency, stable transformants were necessary. An episomal vector was chosen to avoid the biases introduced by subcloning transformed cells with integrated constructs. We show that a fragment extending from bases –370 to +30 of the SI promoter is sufficient to confer glucose-dependent regulation on the reporter gene. We also report evidence that elements in the SI promoter influence cellular glucose consumption.

MATERIALS AND METHODS

Plasmids

The luciferase vectors used for these studies included a promoterless vector (pTZLuc) [26] or the luciferase gene under the

Abbreviations used: EBNA, Epstein–Barr virus nuclear antigen; HNF, hepatocyte nuclear factor; PBS[–], PBS free of Ca²⁺ and Mg²⁺ (2 mM KCl, 1.4 mM KH₂PO₄, 137 mM NaCl, 8 mM K₂HPO₄); SI, sucrase–isomaltase; SSC, 0.15 M NaCl/0.05 M sodium citrate.

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The nucleotide sequence data reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X85797.

-1744 TTGTTAACTA TTTCTCTTGA ACTCATCAGA GAACTAAAA TTAAGGGGAA AACAAATGAT CTGAAATATA AGATTATCA ATGTTGTTGC ATAAATCAGT
 -1644 AGTTTATAAC TTTTCATTGC TGAGTAGTTT GCTATTGTAT GGATATGCCA GAGTTTACAG ACACTTTGTG TGTTTCCAGT TTGGGGAAAT TATCAATAAA
 -1544 GCTGTTATAC AAATTGGTAT AAAAAATTTA CAATCAGCTT GTCTATATTT ACTTCATGGA GTTTTGATTG AGATTATATT TTATGTGTAG ATCTAATAGT
 -1444 GGCAAATTGG CAGCTTAATA CTGAGTTTTC CAGTCTATGA ATATGGCGTA TCTCTCAATT TTTAAAATCT TTAACCTTTT CATCAACATT TTTTGGTGCT
 -1344 ATTCTAATTG ATAATCTAGC TATCTATCAA CTTATATCTC TGTCTATATC TATATTGTAG TTTCAGTTTC CAATTCTTCC TTGCTATTAG GTAGGATCAT
 -1244 GATTGACCTT TGTATGTTGA CCTTATATCC TGTGACCTTG CTTTGCTTGC CTATTGGTTC TAGGAGCTTT GAGTTCACTT AGTGAGATTG TACACATATA
 -1144 AAATAATGTG GCATATGAAT AGAGAGTAAA TCACTCAAAA GTTTTATTAT ATTTTCTCTA TTTAGATCAT TCTGGACTGA TTTTAACTT ATTGCTATAT
 -1044 AACAAATTTT ATATACTGAA ATTTCTCCTT TATATAGCTA CTGAGTCTTA CAACAATCCA TGCATACCTT TAATTTTCTT TTTAGACTCA TATTTAGTTT
 - 944 GCTTCAAAAC TAATTGCTCT ACTTTATGCA AACACATTAT TTTTAATATA TATTTTGTG AAAGATAGTA TCCAGATGGG TATGCAAGGC TTAGACTCTT
 - 844 GACAATTTCA GAGTCAAAT GGGGTCAATA ATAATTAAGC AATATTAGCT CATATAATTC TTTTAGTGAT TTTATAGGTA GTATTTTGCC CACAATTTCT
 - 744 CAAGTAAGCA TAGTACAGCC AGCATTATATA TCCAAATTTA TTATATCAAA TCCATTGCTT TAAACATTA TGCTAGCTTA AACCAGTAGT GTAATTAATT
 - 644 TTATACATTT TACTACATGA AATGAGAAGG TTTACAATAT GATTTTATAT AATTCATGA GTGCTATCTG TGGTAAATAA ATAGATAAAA ATGTGTGATT
 - 544 TATCTCTAAA TTACTGATCT GGGCACCTCT ATTTTATGTT TTATACCTAA GGATTGGTTC ACTTTTTCAT CCTTAAGATC AAAGACAAGA TACAGGGAAT
 - 444 AATTTTATT TAATCATTAC TTTTATAAAA ATATTATCTT CTAATTGTTA TATCTGAAA ATTTTTCACA GATTCTGCTT TTCAAATATG TTTCTACTCT
 - 344 CCAGTGAGGA TCTAAATTGC ACACAATGGA CAAATAGTT ATTATAACAA AATCCAATA TAAATAAAT ATAATTTTGA AAAGAGAAAA AATAGAAAGA
 - 244 ACTACTTTTA TGTCTTAAG ATATTAATAG TTCACAGCTT TGAGAAATCA AAGAGTATCT GACAGTACAA TTACTAATTA ACTTAGATTC CCAGAGAGAA
 - 144 ACATTTATGT AACTACTTT CAGGGTTAAG GCTTTTAAAC ATTGTATGTT GAGCAGAAGA TTATTAACA CTGATAGGCT GGTGAGGGTG CAATAAACT
 - 44 TTATGAGTAG GTCATATAT ACCTAAGCAG CTTTCTTGA TCATTATTTT GGCAGCCTTA TCCAAGTCTG GTAC

Figure 1 Upstream sequence of human SI gene

The position of the major transcription start site was reported previously [3]. The TATA box is underlined. Bold letters designate transcribed bases. The 5' ends of SI H (*Hpa*I, -1739), SI N (*Nhe*I, -672) and SI A (*A*/wNI, -370) are indicated with horizontal arrows. Nucleotide +30 corresponds to the 3' end of SI H, SI N and SI A.

Figure 2 Structure of the plasmids used for transfection experiments

Epstein–Barr virus ori P, EBNA-1 gene and TK promoter (pTK) are required for replication and maintenance in human cells. The hygromycin resistance gene allows selection of the transformed cells. TLuc is the promoterless plasmid. In TSIA, N and H constructs the luciferase gene is under control of the SI A, SI N or SI H fragments of the SI promoter.

control of restriction fragments of the 5' region of the human SI gene (pTZSIXLuc). The different restriction fragments were obtained from a 14 kb human genomic clone [3]. Sequencing was performed on both strands 3 kb upstream from the transcription start sites by the dideoxy chain termination method [27]. Because there were several small differences from the sequence published by Wu et al. [4], the part of the sequence relevant to the present studies is shown in Figure 1. The Luc and SIXLuc inserts were isolated from the corresponding pTZ18R recombinants by digestion with *Pvu*II for subcloning in the *Nar*I/blunt-ended site of a derivative of plasmid p205-GTI [28]. This plasmid contains

the Epstein–Barr virus sequences for ori-P and Epstein–Barr nuclear antigen 1 (EBNA-1) that allow a stable and episomal maintenance of the vector in human cells [29]. The hygromycin resistance gene under the control of the herpes simplex virus 1 (HSV-1) thymidine kinase promoter was used for the selection of transformed cells. The plasmids used for transfections are depicted in Figure 2. They were purified by two successive isopycnic centrifugations in caesium chloride gradients [30].

Cell cultures, transfections and selection of transformed cells

PF11 [25] clone was routinely cultured in Dulbecco's modified Eagle's minimum essential medium containing 25 mM glucose (Eurobio, Paris, France), 20% (v/v) heat-inactivated (56 °C, 30 min) fetal bovine serum (Boehringer, Mannheim, Germany) and 1% (v/v) non-essential amino acids (Gibco, Glasgow, U.K.) and maintained in an air/CO₂ (19:1) atmosphere. For growth in low glucose (1 mM) the same conditions were used, except that the medium was devoid of glucose, the final glucose concentration being supplied by the serum. For transfection experiments, 0.45×10^6 cells were seeded in 25 cm² Petri dishes (Corning Glasswork Co). The medium (5 ml per dish) was changed 2 days later. Transfections were performed 4–5 h after the medium change, by using the CaPO₄ procedure [31] and 20 µg of plasmid DNA per plate. The medium was changed 15 h later and then daily. Hygromycin B was added at confluency at a final concentration of 210 µg/ml, which was determined to be the minimal lethal concentration for the untransformed cells. Surviving hygromycin-resistant cells were allowed to grow until subconfluent and were then subcultured under the following conditions: the cells were allowed to attach to the flask for 48 h without drug and then hygromycin B was added at a concentration increasing progressively from 40 to 210 µg/ml depen-

ding on cell density. In all cases, the medium was changed 48 h after seeding and daily thereafter [25]. During the selection process and in preliminary control experiments, also with another Caco-2 clone, we observed that hygromycin B induces changes in the levels of glucose consumption (A. Rodolosse, I. Chantret, M. Lacasa, G. Chevalier, A. Zweibaum, D. Swallow and M. Rousset, unpublished work). The stably transformed cells were thus subcultured and maintained without the drug for the experiments reported here: the plasmid copy number was shown by slot-blot hybridization analyses to be stable for the 20 days of culture.

Plasmid extraction and DNA analysis of transformed cells

Extrachromosomal and genomic DNA were purified by a small-scale SDS lysis procedure adapted from the method of Birnboim and Doly [32]. DNA extracts were analysed by slot-blot and hybridization with a luciferase probe (a 759 bp *EcoRI*–*KpnI* fragment from PTZLuc) for the plasmids and SI2 [33] for the SI gene. Probes were labelled with ^{32}P by using a megaprime DNA labelling kit (Amersham). After 18 h of hybridization in the presence of 10% (w/v) dextran sulphate (Pharmacia) the blots were washed twice in $2 \times \text{SSC}/0.1\%$ SDS at 65°C for 30 min and then for 15 min in $0.1 \times \text{SSC}$ at 65°C (where SSC is 0.15 M NaCl/0.05 M sodium citrate). Quantification was achieved by densitometric scanning (Mark III CS; Joyce, Loeb and Co, Ltd, Gateshead, Tyne and Wear, U.K.) of different film exposures to achieve conditions of linearity.

Luciferase assays

Cell layers were rinsed twice in PBS[−] (PBS free of Ca^{2+} and Mg^{2+} , consisting of 2 mM KCl, 1.4 mM KH_2PO_4 , 137 mM NaCl and 8 mM K_2HPO_4) and frozen for 1 h at -20°C . After thawing on ice, the cells were scraped from the surface of the flasks in lysis buffer (15% glycerol, 25 mM Tris, 8 mM MgCl_2 , 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, PMSF 0.4 mM). The extract was frozen at -20°C for 1 h, thawed and centrifuged for 15 min at 25000 g rev./min at 4°C . (Microfuge 2, Beckman). Supernatant (20 μl) was used for the enzymic determination with a luciferase assay kit (Promega). The activity was estimated in a Lumat LB9501 luminometer (Berthold). Protein concentration was assayed with the BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.). Luciferase activities are expressed as light units/s per μg of protein per plasmid copy. Light units correspond to the counted pulses divided by 10.

RNA extraction and analysis

Total RNA was extracted by the guanidium isothiocyanate method [34]. Samples of total RNA, denatured in 1 mM glyoxal, were fractionated by electrophoresis through 1% (w/v) agarose gels and transferred onto Hybond N (Amersham) [35]. Pre-hybridization was performed at 42°C in the presence of 50% (v/v) formamide, and hybridization at 42°C in the presence of 40% (v/v) formamide and 10% (w/v) dextran sulphate with the luciferase or SI2 probes. The blots were washed twice in $2 \times \text{SSC}/0.1\%$ SDS for 10 min at room temperature, once in $0.1 \times \text{SSC}/0.1\%$ SDS for 15 min at 50°C and once in $0.1 \times \text{SSC}/0.1\%$ SDS for 15 min at 65°C .

Immunofluorescence

Indirect immunofluorescence was performed on the unpermeabilized cell layer or on frozen cryostat sections from the same

cultures as described previously [21] after fixation in 3.5% (w/v) paraformaldehyde in PBS[−] for 15 min at room temperature. Monoclonal antibodies against human intestinal SI (HBB 2/614/88) were obtained from Dr. H. P. Hauri (Biocenter of Basel University, Basel, Switzerland) [36] and rabbit polyclonal antibodies against porcine villin were obtained from Dr. D. Louvard (Institut Pasteur, Paris, France) [37]. The anti-mouse and anti-rabbit fluorescein-coupled sheep antiglobulins were from Institut Pasteur Productions (Marnes-la-Coquette, France).

Glucose consumption assays

Glucose consumption was determined every day 16–18 h after the medium changes in two independent flasks by using a Beckman Glucose Analyser 2. Results are expressed as nmol/h per 10^6 cells.

Sucrase activity assays

Sucrase (EC 3.2.1.48) activity was measured on the cell homogenates by the method of Messer and Dahlqvist [38]. Results are expressed as nmol/min per mg of protein.

RESULTS

Glucose consumption

When cultured in the absence of hygromycin and in the presence of 25 mM glucose, all the transfectants showed high glucose consumption. The promoterless PF11-TLuc and the TSI H and TSI N populations (Figure 3) showed levels of glucose consumption that were indistinguishable from those determined for the untransformed cells, whereas in the PF11-TSI A population, which contained the smallest $-370/+30$ construct, the values measured from day 10 to day 20 were half of those obtained for the three other constructs. As the rate of glucose consumption is not affected by the extracellular glucose concentration, this meant that when cultured in 1 mM glucose, the extracellular glucose disappeared within 2 h for PF11-TLuc, TSI H and TSI N cells and within 4 h for PF11-TSI A cells after a medium change in post-confluent cells (results not shown).

Figure 3 Growth-related glucose consumption of PF11 transformed cells

Results are means $\pm$ S.D. for two independent flasks of two separate cultures in 25 mM glucose, without hygromycin, of PF11-TSI A ($\blacklozenge$) of PF11-TLuc ($\blacksquare$) cells. Similar results were obtained for the TSI H and TSI N constructions as for TLuc.

Figure 4 Indirect immunofluorescence detection of SI and villin in post-confluent (day 18) PF11-TSI A cells

The cells were cultured in 1 mM (**a, c** and **e**) or 25 mM (**b, d** and **f**) glucose. SI was detected on the cell surface (**a, b**) or in cryostat sections of the cell layer (**c** and **d**). Villin (**e** and **f**) was detected in serial sections from the same samples. Scale bar = 27 μ m. Similar results were obtained with other constructs.

Endogenous SI expression

The presence and localization of the SI protein was studied by immunofluorescence staining of the transformed cells cultured in either 25 mM or 1 mM glucose. With the non-permeabilized cell layer (Figures 4a and 4b) the number of positive cells as well as their fluorescence were considerably greater with 1 mM than with 25 mM glucose. Results obtained on sections of the cell layers showed that whereas villin was expressed at the apical pole of all the cells at both glucose concentrations (Figures 4e and 4f), the SI protein was scarcely detected on cells cultured in 25 mM glucose (Figures 4c and 4d). The protein, when present, was always localized in the apical membrane; no signal could be detected in the cytoplasm of the transformed cells under either set of culture conditions.

Similar differences were also observed for endogenous sucrase enzyme activities (results not shown) and at the RNA level (Figure 5a). At both high and low glucose concentrations a similar growth-related increase in SI mRNA is observed for all

the constructions as previously reported for the untransformed cells. In all cases and as early as on day 10, the SI mRNA was more abundant in 1 mM than in 25 mM glucose (Figure 5a). However, in 25 mM glucose the SI mRNA levels increased more rapidly in the less glucose-consuming PF11-TSI A transfectants than in the cells harbouring the other constructs (Figure 5a).

Expression of the reporter gene luciferase

The luciferase activities were measured between day 10 and day 20 for the promoterless control and the three constructs for which the gene was under control of the SI promoter. All three promoter-containing constructs showed much higher luciferase expression than the promoterless control. In each case, notably including the smallest construct TSI A, the enzymic activity was systematically 2–4-fold greater with 1 mM than with 25 mM glucose between days 10 and 14 (Table 1). In all cases a marked decrease in the luciferase activity was observed between day 14 and day 20.

Figure 5 Northern-blot analysis of SI and Luc mRNA in PF11-transformed cells

The cells were cultured for 20 days in 1 or 25 mM glucose. Total RNA (20 μ g) was loaded in each lane. The same filter was hybridized either with the SI2 cDNA probe (a) or with the Luc probe (b). Note that the non-specific hybridization to the 18 S ribosomal RNA band that can be seen with the Luc probe was of the same intensity in all lanes. SI and Luc signals correspond to 24 h or 48 h autoradiographic exposures respectively. Signals obtained with the SI probe for TSI H and TSI N were similar to those shown for TLuc in (a). Panel (b) is representative of the results obtained with the TSI H, TSI N or TSI A construct.

Table 1 Glucose-dependent luciferase activities in PF11 transformed cells

Fold increase over promoterless TLuc of luciferase activities from day 10 to day 20 of PF11-TSI H, TSI N and TSI A cells cultured in 25 or 1 mM glucose. Results are means $\pm$ S.D. for two determinations in two separate experiments.

Construct ... Glucose concentration (mM) ...	TSI H		TSI N		TSI A	
	25	1	25	1	25	1
Day 10	28 $\pm$ 4	50 $\pm$ 6	28 $\pm$ 3	50 $\pm$ 6	45 $\pm$ 6	80 $\pm$ 9
Day 12	38 $\pm$ 4	80 $\pm$ 9	25 $\pm$ 3	95 $\pm$ 10	35 $\pm$ 4	98 $\pm$ 10
Day 14	45 $\pm$ 5	90 $\pm$ 10	26 $\pm$ 3	98 $\pm$ 10	34 $\pm$ 4	120 $\pm$ 11
Day 20	12 $\pm$ 2	26 $\pm$ 4	12 $\pm$ 2	24 $\pm$ 3	12 $\pm$ 2	26 $\pm$ 4

Figure 5(b) shows the luciferase mRNA expression. Although difficult to detect, the 1.8 kb luciferase transcript was always more abundant with 1 mM than with 25 mM glucose. Maximal levels were seen on days 12 and 14, but by day 20 the luciferase-specific mRNA could no longer be detected at either glucose concentration. Similar results were obtained for TSI H, TSI N and TSI A constructs, but with the TLuc control construct no signal at all could be detected at any stage or at either glucose concentration.

DISCUSSION

This study was undertaken as a first step towards the characterization of regulatory elements responsible for the glucose-dependent repression of the human SI gene transcription [3,18,20–25].

The results reported here show that the $-370/+30$ (SI A) region of the human SI gene promoter is able to confer glucose-repressible expression on a heterologous gene: a decrease of the luciferase expression driven by the SI promoter was observed when PF11 cells transformed with all three constructs were cultured in 25 mM instead of 1 mM glucose. The variations in luciferase activities were always lower than those observed for the endogenous SI but these differences are most probably due to the short half-lives of the luciferase mRNA and protein (about 6 and 3 h respectively) [39] compared with those of SI (30 h for the mRNA [24] and more than 3 days for the protein [21]). The marked decrease in the reporter gene activity between day 14 and day 20 in the PF11 transformed cells (Table 1) could reflect the slight increase in glucose consumption that occurs at this stage (Figure 3), an effect that would be difficult to observe with a stable protein. It remains to be determined whether the same genomic region is responsible for the growth-related increase in SI expression, which coincides with a decrease in glucose consumption in differentiating cultured cells, and whether it is also involved in all the various situations of catabolic glucose repression of SI in both Caco-2 and HT-29 cells [18,21,22,24].

Another complexity is evident from the results obtained from the transfections with the TSI A construction: PF11-TSI A cells cultured in 25 mM glucose were found to have systematically lower levels of glucose consumption than the cells transformed with the other constructs, including the promoterless control. The smallest construct shows the highest luciferase expression and higher endogenous SI expression. The molecular mechanisms by which this occurs are not clear and it seems probable that the effect is due to the presence in the cells of multiple copies of the relevant sequences rather than it being of 'in vivo' significance itself. However, the results are intriguing and clearly show that the adjacent segment ($-672/-370$) contains an element that specifically reverses the effect. Nothing is yet known about the DNA-protein interactions in this region but this clearly merits further investigation.

Taken together, the results reported here show that the glucose-dependent regulation of SI gene transcription involves complex mechanisms related in part to elements that affect the glucose consumption of the cells as well as to elements responsible for the repression of transcription by glucose. As previously discussed [3] we found no sequence homology between the upstream region of the SI gene and those of the human Glucose Regulated Proteins [40] or those of glucose repressible genes in yeast [41]. We also failed to detect the element responsible for the induction by glucose of genes encoding hormones or enzymes involved in the glucose metabolism ([42,43] and references therein).

The $-370/+30$ SI A region contains three nuclear protein-binding sites that have already been characterized: SIF1 ($-53/-37$) is responsible for intestine-specific SI transcription [8,44]; SIF2 ($-86/-73$) and SIF3 ($-176/-156$) were found to bind hepatocyte nuclear factor 1 β (HNF-1 β) (vHNF-1) and HNF-1 α [7,44]. It is of interest that one of the three HNF-1 β proteins (vHNF-1c), unlike all other members of the HNF family described so far, behaves as a transdominant repressor instead of an activator [45]. Furthermore in rat kidneys the phosphoenolpyruvate carboxykinase gene, which is normally repressed by glucose, was unresponsive to a carbohydrate diet when its HNF-1 binding motif was mutated [46]. The possibility that homo- or hetero-multimers of the HNF-1 homeoprotein family [47] play a role in the glucose repression of the SI gene is currently under study.

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