The complete hGSTP1*C gene, consisting of 7 exons and 6 introns contained in 3116 base pairs, was isolated from a cosmid genomic library of a glioblastoma multiforme cell line. Although the promoter of hGSTP1*C was identical to that of the previously reported GST-Pi gene, several of its structural features had not been previously described. These include several nucleotide transitions and transversions. Transitions of A → G at c.1404 and C → T at c.2294 in exons 5 and 6, respectively, changed codons Ile52 to Val154 and Ala113 to Val113. The gene also contained a guanine insertion at +51 in the insulin response element in intron 1 and six tandem repeats and one palindromic retinoic acid response element (RARE) consensus half-sites, A(G/GG(T)C/G)A in intron 5. Retinoic acid (RA) treatment increased GST-Pi gene expression significantly in MGR3 cells. GST-Pi gene constructs with and without RARE deletion were used to show the RARE requirement for GST-Pi gene induction by RA. The isolation of the hGSTP1*C gene and the evidence that it contains functional RAREs should contribute to a better understanding of the molecular regulation of the GST-Pi gene in human cells.

The glutathione S-transferases (GSTs) are best known for their ability to catalyze the neutrophilic attack of the sulfur atom of glutathione by a variety of electrophilic endogenous and exogenous compounds (1–5), producing water-soluble and often less reactive metabolites. Much interest is currently being focused on the Pi class GST because the gene is up-regulated during the early stages of oncogenesis and it is the most significantly overexpressed GST gene in many human tumors including gliomas (6–17). A large number of studies have also shown the high levels of GST-Pi expression to be associated directly with tumor drug resistance and with poor patient survival (9, 12, 16–19). In gliomas, the level of GST-Pi expression correlates positively with tumor grade (15, 20), and in glioma cell lines, high GST-Pi expression has been correlated with increased resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (12).

Recently, we reported the isolation of three full-length cDNAs, hGSTP1*A, hGSTP1*B, and hGSTP1*C, corresponding to closely related GST-Pi mRNAs and encoding structurally and functionally different GST-Pi proteins (21). Of the three allelic GST-Pi gene variants, hGSTP1*C was shown to be expressed at a much higher frequency in malignant gliomas than in normal brain, placenta, and lymphocytes. To facilitate studies aimed at clarifying the molecular mechanisms underlying the overexpression of the GST-Pi gene in human gliomas, we undertook in this study the isolation and characterization of the hGSTP1*C gene from cells of a human glioblastoma multiforme cell line that expresses high levels of GST-Pi gene transcripts and protein and is resistant to 2-chloroethyl-nitrosoureas and cisplatin. We compared the isolated gene with the previously described GST-Pi gene and examined its regulation by all-trans retinoic acid (RA).

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases, Klenow enzyme, and T4 DNA ligase were purchased from Boehringer Mannheim. Proteinase K, RNase A, and all-trans RA were from Sigma. SuperCos 1 cosmid vector, Bluescript phagemid II, Gigapack II packaging system, calf intestinal alkaline phosphate, pBK-CMV expression vector, and the methylsulfonylbenzene mammalian transfection kit were purchased from Stratagene, La Jolla, CA. [35S]dATP and [α-32P]dCTP were purchased from Amer sham Life Science, Inc. Taq DNA polymerase was purchased from Perkin-Elmer. Dulbecco’s modified Eagle’s medium and fetal calf serum were from Life Technology, Inc. T7 DNA Sequenase 2.0 dyeoxy DNA sequencing kit was purchased from U. S. Biochemical Corp.

**Tumor Cells—**The MGR3 human glioblastoma multiforme cell line was established in our laboratory from a primary specimen, as described previously (22). It is glial fibrillary acidic protein positive by immunocytochemistry, and the cells show the typical pleomorphism of neoplastic glial cells. The cell line is routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and nonessential amino acids and had undergone 11 in vitro passages before being used in these studies. MCF7, obtained from the American Type Tissue Collection, Gaithersburg, MD, is a human mammary carcinoma cell line that, under normal physiological conditions, does not express detectable basal levels of GST-Pi transcripts or protein.

**Southern and Northern Blotting—**These were performed using standard techniques (23). For Southern analysis, genomic DNA was extracted from MGR3 cells using the phenol/chloroform procedure (23), and for the Northern blots, total RNA was isolated with the acid guanidinium thiocyanate/phenol/chloroform procedure (24). The 100-μl reaction mixture contained 50 ng of SuperCos-GST-Pi or other DNA template, 500 ng each of forward and reverse primers, 10 × PCR buffer, 100 mM each dATP, dCTP, dGTP, and dITP. 2.5 units of AmpliTaq polymerase was added, and the mixture was overlaid with mineral oil. After 1 cycle of denaturing (95°C for 90 s),
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**Table I**

| Amplified region of GST-Pi gene | Amplification primers | Fragment size (bp) |
|---------------------------------|-----------------------|-------------------|
| Exons 2–3                        | P1: GCGAAGCTTCGACCACCTGGCCGGCCTTC | 430 |
|                                  | P2: GAGGTGTCGACGGAACCTGCAA | |
| Exons 3–6                        | P1: AGATCAAGCCCCAGACGGCTGAAG | 1,700 |
|                                  | P2: CTGGTCTGGGACAGGCACCTGTC | |
| Exon 7–poly A site               | P1: CTTCGTCTTAGAGGGACGGA | 940 |
|                                  | P2: TCTTCCCTCTTATTGTTGAGG | |
| Intron 5                         | P1: CAGGCCCTTCGACATGGTCGAA | 1,000 |
|                                  | P2: CTGTCCTGGGACAGGCACCTC | |
| Intron 6                         | P1: TGGCACTGGATAGTGAGGATT | 450 |
|                                  | P2: GATGCAGCAAGATGCCCAGCAG | |

**Results**

**Construction and Screening of Genomic Library—Approximately 10^8** colonies of the MGR3 SuperCos 1 genomic library were initially screened with the GST-Pi cDNA probe. Twenty positive colonies were subjected to secondary screening, after which two were selected for tertiary screening. One positive clone designated SuperCos-GST-Pi was selected for further analysis.

**Restriction Endonuclease Mapping of SuperCos-GST-Pi**

The results of Southern analysis of NotI and HindIII-digested SuperCos-GST-Pi with a GST-Pi cDNA probe were used to construct a simplified restriction map of the SuperCos-GST-Pi clone. The map showed the GST-Pi gene to be located between two NotI sites of the SuperCos-GST-Pi clone and to overlap two HindIII fragments. The entire gene was contained within a 2.1-kb NotI-HindIII fragment and an 11.5-kb HindIII fragment. This fragment was subsequently verified by computer analysis of the final DNA sequence.

**Nucleotide Sequence and Structural Analysis**

The nucleotide sequence of the isolated GST-Pi gene was shown in Fig. 1. The sequence was compared with those of the previously described GST-Pi genes from the MCF7 cell line (1) and the MCF7-23 cell line (2) respectively. For further analysis of exons 5 and 6 of the isolated GST-Pi gene, a 1-kb DNA fragment containing intron 5 and a 450-bp fragment containing intron 6 as well as the regions flanking these introns were amplified using the primer pairs listed in Table I. The PCR products were purified and digested to confirm the presence of SpeI and AvalI restriction sites in introns 5 and 6, respectively. The entire gene and its promoter could be regulated by retinoic acid (28, 29), we analyzed the cloned GST-Pi gene for the presence of sequences homologous to known retinoic acid response element (RARE) consensus sequences (30).

**GST-Pi Expression Vector Construction and Expression**

The complete GST-Pi gene was obtained by ligating a 2.2-kb NotI/BamHI fragment to a 0.9-kb BamHI/KpnI fragment, both from

SuperCos-GST-Pi into the NotI/KpnI site of the pBK-CMV vector (Stratagene) in which eukaryotic expression is driven by the cytomegalovirus (CMV) immediate early promoter. The resulting GST-Pi expression construct, designated pGST-Pi-CMV, contained the entire 3.1-kb GST-Pi gene consisting of 131 bp of 5′ promoter region, 109 bp of 3′ untranslated region including the polyadenylation signal, and 68 bp downstream of the polyadenylation signal.

pGST-Pi-CMV was transfected into exponentially growing Cos 1 cells using the calcium phosphate method (23). After 48 h, the cells were harvested, washed twice in phosphate-buffered saline, homogenized, and centrifuged at 20,000 × g for 20 min at 4 °C. Protein concentrations and total GST enzyme activity in the supernatants were determined as described previously (12, 31), the latter using 1-chloro-2,4-dinitrobenzene as substrate. Specific GST-Pi protein content was determined by Western blotting as we had previously described (12).

**RA Effect on GST-Pi Gene Expression in Glioma Cells**—These studies were performed to examine the responsiveness of the GST-Pi gene to RA. Exponentially growing MGR3 cells from which the GST-Pi gene was isolated were treated with 1 μM all-trans RA, and after 24 and 48 h of incubation at 37 °C, total RNA and protein were extracted from control and RA-treated cells, and GST-Pi gene transcript and protein levels were determined by Northern and Western blotting, respectively, as we described earlier.

**RA Effects on Full-length and RARE-deleted Constructs of GST-Pi Expression Vectors**—To further characterize the functionality of the RAREs in the cloned GST-Pi gene, the RA response of the expression vector, pGST-Pi-CMV, containing the full-length AGSTPi°C gene and of one, pGST-Pi-CMV-RARE (−), containing the hGSTPi°C gene from which the RARE region had been deleted, were examined. The latter vector was obtained by Bso3I digestion of the pGST-Pi-CMV to release a 476-bp fragment containing all seven RARE half-sites. After blunt-ending with DNA Kleng enzyme, the linear plasmid was religated to create the circular expression vector.

Exponentially growing MCF7 cells were transfected with pGST-Pi-CMV-RARE (−), pGST-Pi-CMV, and parent pBK-CMV (without the GST-Pi gene). The cells were then treated with 1 μM all-trans RA. Control cells transfected with the vectors were similarly set up but without RA treatment. Forty-eight hours later, the cells were harvested and examined for GST-Pi gene transcripts by Northern analysis. The rationale for using MCF7 instead of MGR3 for these studies is that the GST-Pi gene is transcriptionally silent in MCF7 cells and is not induced by RA. As such, any differences in the GST-Pi gene expression following RA treatment of transfected cells can be attributed to RA effects on the transfected vectors. In contrast, MGR3 cells express high basal GST-Pi levels, and RA treatment results in a significant increase in GST-Pi gene expression, thus making it difficult to distinguish the differential effects of RA on the RARE-deleted and -undetected vectors after transfection.

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isolated GST-Pi gene consists of 211 codons including the ATG initiation and TGA termination codons. The 3′ noncoding region of the gene covers nucleotides 12763 to 12984 and includes the polyadenylation signal AATAAA at 12818 to 12823.

The 5′-flanking region upstream of the first exon of the gene, i.e., the promoter region, contains five regulatory motifs, all of which have been previously reported (26, 27). Relative to the transcription initiation site (26), these were a TATAA box located at 231 to 227, two Sp1 sites at 246 to 241 and 257 to 251, and an AP-1 site at 269 to 263. Embedded in the AP-1 site at 270 to 261 was an antioxidant response element (ARE) core consensus sequence.

Comparison of Isolated GST-Pi Gene with Previously Described Human GST-Pi Genes—Table II summarizes the structural differences between the GST-Pi gene isolated from the MGR3 cell line and the previously reported GST-Pi gene from the MCF7 cell line (27). Two transitions, an A to G at 1140 and a C to T at +2294 changed codon 104 from ATC (Ile) to GTT (Val) and codon 113 from GCG (Ala) to GTG (Val), respectively. These findings confirmed the isolated gene to be hGSTP1*C, the full-length cDNA of which was recently isolated in our laboratory (21).

In addition to the three nucleotide transitions in exons 5, 6, and 7, several intronic differences were also observed between the MGR3 and the MCF7 GST-Pi genes. A region of high homology with the core sequence (CCCGCCTC) of the insulin response element A, IRE-A (32) was observed at 145 to 152. This IRE differed from that previously described at the same location in the GST-Pi gene isolated from the MCF7 (27) by having a guanine insertion at 151. The insertion created two CpG dinucleotides and a cleavage site (CGCG) for several restriction endonucleases, including AccII, AciI, Bsp50I, BstUI, and MvnI. Additionally, nucleotide transitions in introns 5 and 6 created extra endonuclease cleavage sites in the MGR3 GST-Pi gene that are absent in the MCF7 gene. A G to A transition at nucleotide 11968 created a SpeI site, AGTG, in intron 5, which was confirmed by SpeI digestion of a 1-kb PCR product containing intron 5. Interestingly, only partial cleavage was observed upon SpeI digestion of the same DNA region from human lymphocytes and MCF7 cells, indicating the existence of polymorphism of the GST-Pi gene at this position. Additionally, two transitions of A to G and C to T at +2557 and +2559, respectively, created within intron 6 new AvaII cleavage sites GGTCC that are not present in the MCF7 gene.

![Fig. 1. Nucleotide and deduced amino acid sequences of the complete GST-Pi gene. The AP-1 and SP-1 sites, TATAA box, ARE, IRE, RARE, and the AATAAA polyadenylation signal are underlined. AG/GT splicing signals (underlined) were used to determine intron/exon boundaries. Codon changes are in boldface. Overlapping fragments of the GST-Pi gene were cloned into pBluescript phagemid II and sequenced by the 35S-dideoxynucleotide method.](image-url)
Summary of structural differences between GST-Pi gene isolated from MGR3 glioblastoma multiforme cell line (GenBank™ accession number U21689) and hGSTP1*A, previously isolated from the MCF7 cell line (GenBank™ accession number X08058)

| Cell line | Intron/exon (nucleotides) | Sequence | Structural change, modified endonuclease cleavage site |
|-----------|---------------------------|----------|-------------------------------------------------------|
| MGR3      | Intron 1 (+51)            | CGCTGC   | Guanine insertion;                                      |
| MCF7      | Intron 2 (+556)           | CCCTCG   | CG | CG: AccII, AccI, Bsp50I, BstUI, MunI               |
| MCF7      | Intron 3 (+1,968)         | TACTAG   | A → C transversion                                     |
| MCF7      | Intron 5 (+2,559)         | ACTAGG   | No known endonuclease site modification                 |
| MCF7      | Intron 6 (+2,557, +2,559) | CCTGTGCCC| A → G transition                                       |
| MGR3      | Exon 5 (+1,404)           | GTCTGC   | A → G transition                                       |
| MCF7      | Exon 6 (+2,294)           | ATCTGC   | ATC (Ile) → GTC (Val) at codon 104                     |
| MCF7      | Exon 7 (+2,684)           | GGTGAG   | GCC (Ala) → GTC (Val) at codon 113                     |
| MGR3      | Exon 7 (+2,684)           | AGCAGT   | T → C transition (silent polymorphism)                 |
| MCF7      | Exon 7 (+2,684)           | AGTAGT   | AGT (Ser) → AGC (Ser) at codon 184                     |

Table II

Expression of Cloned GST-Pi Gene in Cos-1 Cells—The structure of the pGST-Pi-CMV expression vector is shown in Fig. 2, and the Western blot analysis for GST-Pi protein in control Cos-1 cells and in Cos-1 cells 48 h after transfection with the pGST-Pi-CMV vector is shown in Fig. 2b. Densitometry of the Western blots showed a 2.9-fold-increased GST-Pi protein content in the transfected cells relative to controls. The similar levels of increase in total GST activity and specific GST-Pi content indicate that the increase in GST enzyme activity was due, primarily, to the overexpressed GST-Pi protein.

RAREs in Isolated GST-Pi Gene—RAREs are direct repeat regulatory motifs to which RA-RAR complexes bind and mediate transcription of RA-responsive genes (30, 34–36). We report for the first time the presence of RARE sequences in the GST-Pi gene. These RARE motifs are located in intron 5 of the GST-Pi gene, in a region spanning nucleotides +1521 to +1644 and consists of one palindromic and six direct repeats of RARE consensus half-sites arranged in tandem. Fig. 3 shows the region of the GST-Pi gene with the six direct repeats and one palindromic RARE half-sites, and Table III compares the consensus RAREs in the isolated GST-Pi gene with known RAREs in other selected genes.

RA Effects on Cellular GST-Pi Gene Expression and on Expression of RARE-containing and RARE-deleted hGSTP1*Gens in Expression Vectors—The results of these studies designed to examine response of the GST-Pi gene to RA are summarized in Fig. 4, a and b. Northern analysis (Fig. 4a) showed a moderate but significant increase in the level of
The RARE half-sites are in boldface. Spacer regions with greater than six nucleotides are designated by the number of nucleotides. N, nucleotides.

| Gene                  | RARE consensus half-site sequences |
|-----------------------|-----------------------------------|
| GST-Pi                | TGACCC CTTCTT                      |
| GGGACA                | 26N GGGACA 13N GGGACA 17N GGGTGCA |
| RARα2                 | GGGTCA TTCAG AGTCTC               |
| RARγ2                 | GGGTCA CCGAA AGGGC                |
| ApoAI                 | GGGTCA AG GGGTCA                 |
| ApoCII                | TGGGCA A AGTCTC                  |
| Phosphoenolpyruvate   | CCGGCA A AGTCTC                  |
| carboxykinase         |                                   |
| Oxytocin              | GGGTCA AGTCTC                    |
| Cellular retinol binding protein I | AGGGTTAA |
| GGGTCA                | AGTCTC AA AAGTTA                  |
| Cellular retinol binding protein II | AGGGTTA  |
| AGGGTTA               | AGTCTC kC AGGTTA 14N GGGGCA      |
| Laminin B1            | AGGGTTA 11N GGGGCA               |
| ADH3                  | AGGGTTA AGGGTTA                 |
| Oct-3/4               | AGGGTTA 28N AGGGTTA 10N AGGGGA |
| Oct-4                 | AGGGTTA                         |

**DISCUSSION**

The human GST-PI gene locus has now been shown conclusively to be polymorphic (21). Of the three allelic gene variants, however, only one, hGSTP1*A, has been isolated (26, 27). We describe here the cloning and the structural and functional characterization of another human GST-Pi gene, hGSTP1*C, that in preliminary studies has been shown to be expressed at an increased relative frequency in malignant gliomas compared with normal lymphocytes. The sequenced region of the isolated clone consisted of 3116 nucleotides and contained the complete GST-Pi gene consisting of seven exons and six introns and encoding 210 amino acids. Several of the structural features previously observed in the hGSTP1*A gene isolated independently by Cowell et al. (26) and by Morrow et al. (27) were present in the isolated hGSTP1*C gene. These include a TATAA box, two SP-1 sites, and one AP-1 site in the promoter region. An anti-oxidant response element, ARE, was also observed in the AP-1 site of the isolated hGSTP1*C gene. This ARE, also recently observed in the GST-Pi*A gene (37), is identical to the ARE core sequence (GTGACTCAGC) of the human NADPH:quinone oxidoreductase gene (3, 38) and has a high degree of homology to the ARE (GTGACAAAGC) in the rat GST-Ya gene (3, 39).

Several important differences were observed between the GST-Pi gene described here and that previously reported (26, 27). These include nucleotide transitions of A → G at +1404
The RAREs in the cloned GST-Pi gene are functional and are essential for protein binding and function of the RAREs (30). We performed a number of experiments to demonstrate that the GST-Pi gene isolated from the HPB-ALL cell line (26) and the GST-Pi gene reported in other studies include transitions in introns 5 and 6 that resulted in altered restriction enzyme cleavage sites in the affected regions. These changes do not involve any known regulatory motifs, and as such may have no direct functional consequences, although they may be useful in the characterization of the GST-Pi gene in different individuals. In contrast, the guanine insertion at +51 in the conserved IRE (CCCGGCCTC) in intron 1 is of potential functional significance. This IRE differs from the IRE in the GST-Pi gene isolated from the MCF7 cell line (27) and the IRE in the human glyceraldehyde-3-phosphate dehydrogenase gene (33), which has a C → G transversion at +51. The hGSTP1*C IRE is, however, identical to the IRE previously described in the GST-Pi gene isolated from the HPB-ALL cell line (26) and shown in a chloramphenicol acetyltransferase plasmid construct to be insulin-responsive (28). It remains to be established whether the altered IRE is a common feature of the GST-Pi gene in human glioma cells and/or whether the different IREs have differential insulin binding and ultimately could result in variable insulin responsiveness of the GST-Pi gene. A further significance of the guanine insertion in the hGSTP1*C IRE is that it created two CpG dinucleotides, potential sites of 5-cytosine methylation, which either directly or indirectly through altered insulin binding could result in differential regulation of the GST-Pi gene in different tumors.

In this study, we report for the first time the presence of RAREs in the human GST-Pi gene. The six direct repeat and one palindromic RARE half-sites were all located in intron 5 and are highly homologous to the consensus RARE half-site, 5'-AGGTTTCGA-3', present and functional in other RA-responsive genes, such as those encoding RAR types α2, β2, and γ2 (Table III; 30, 34, 40–42) and the cellular retinoic acid and retinol binding proteins (34, 43, 44, 54, 55), the alcohol dehydrogenase gene (45), and the laminin B1 gene (46). Two pairs of the hGSTP1*C RARE half-sites were separated by the numbers of nucleotides, which in previous studies were shown to be essential for protein binding and function of the RAREs (30). We performed a number of experiments to demonstrate that the RAREs in the cloned GST-Pi gene are functional and are involved in RA-mediated induction of GST-Pi gene expression. We showed that transcription of the isolated GST-Pi gene could be induced with retinoic acid after transfection into tumor cells. Furthermore, following all-trans RA treatment, expression of the GST-Pi gene was increased by approximately 3-fold in MGR3 cells from which the gene was isolated. Using eukaryotic expression vector constructs containing the GST-Pi gene with and without deletion of the RARE region, we demonstrated that the RARE motif was required for the transcriptional activation of the GST-Pi gene in cells treated with all-trans RA.

It was of interest that the RARE region in the hGSTP1*C gene, although intronic, was functional and could regulate RA response of the GST-Pi gene. This contrasts with the majority of previously described RAREs, which are cis-acting and are located in the 5'/promoter regions of the genes they regulate. Functional regulatory motifs within introns, as we observed for both the IRE and the RAREs in the hGSTP1*C gene, are however not unusual in eukaryotic genes and have been shown in a variety of other genes, including oncogenes, tumor suppressor genes, and genes encoding growth factors and their receptors (47–53). A number of these intronic regulatory elements are located distant from the transcription initiation sites of the regulated genes. In the hGSTP1*C gene, the RARE region is approximately 1,500 bp from the promoter site, a distance similar to that of the tissue-specific regulatory element in the p53 gene (49) and those of the two negative regulatory elements of the PDGF-A chain gene (50) from their transcription start sites.

The observations in this study that RA increases GST-Pi expression in tumor cells transfected with the hGSTP1*C gene containing the RARE but not with a gene in which the RARE is deleted contrast with the results of a previous study that showed RA-mediated suppression of the GST-Pi promoter in a chloramphenicol acetyltransferase cDNA construct (28) and suggest a complex mechanism of cellular GST-Pi gene regulation based in part on the previously described antagonism between the AP-1 site and RA gene induction (56–58). In this model of gene regulation, suppression of the GST-Pi gene occurs via competitive inhibition of the binding of AP-1-binding transcription factors such as jun and fos to the AP-1 site by the RA-RAR complex, as has also been suggested in a previous study (28). GST-Pi gene activation, on the other hand, will be mediated through the binding of the RA-RAR complex to RAREs in the GST-Pi gene. Such a model is also consistent with a previously proposed general mechanism for RA-mediated gene regulation by ligand-activated RARs (58). It is further supported by the fact that the activation of the GST-Pi gene by all-trans RA in MGR3 cells observed in this study is a delayed process similar to the late transcriptional induction of the laminin B1 gene by RA (59) and consistent with a mechanism involving RAR-ligand binding to RAREs.

The ability of RA to activate the GST-Pi gene has significant implications for both cancer prevention and chemotherapy. It suggests that part of the molecular basis for the cancer preventive action of long term administration of retinoic acid (60) may involve induction of GST-Pi gene expression. Given the important role that GST-Pi plays in the ability of tumor cells to inactivate anticancer agents, these results also suggest that RA pretreatment is likely to increase tumor resistance to alkylating agent chemotherapy. The isolation of a complete variant GST-Pi gene is an important step in the study of this gene and should facilitate future studies on its molecular regulation in both normal and neoplastic cells.

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