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ASIMILACION DE AMONIO Y BIOSINTESIS DE GLUTAMATO EN *Saccharomyces cerevisiae* y *Kluyveromyces lactis*

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A

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PAGINACIÓN

DISCONTINUA

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CONTENIDOS

RESUMEN

La asimilación de amonio y la biosíntesis de glutamato en la levadura *S. cerevisiae* se llevan a cabo a través de la enzima glutamato deshidrogenasa ($NADP^+$-GDH1) y del ciclo formado por las enzimas glutamino sintetasa y glutamato sintasa (GS-GOGAT). En el presente trabajo se caracterizan estas rutas metabólicas y se describe la presencia de otra enzima que puede llevar a cabo la misma función: la proteína $NADP^+$-GDH3.

La caracterización bioquímica de la enzima GOGAT, la clonación del gen *GLT1* que la codifica y la obtención de mutantes estructurales *glt1*, permitió determinar la existencia de una sola enzima con actividad de GOGAT en esta levadura. La comparación de la secuencia de aminoácidos de esta proteína con la reportada para enzimas GOGAT de otros organismos, mostró que los dominios funcionales y la organización estructural de esta proteína se encuentran altamente conservados, presentando mayor similitud con la enzima de eucariontes superiores.

Los niveles celulares de la enzima GOGAT de *S. cerevisiae* están regulados a nivel transcripcional por la fuente de nitrógeno. La expresión del gen *GLT1* está regulada negativamente por glutamato, el producto de la reacción de GOGAT. En condiciones de exceso de glutamato, la expresión de *GLT1* está gobernada tanto por represión mediada por glutamato, como por activación llevada a cabo por Gln3p y Gcn4p. Cuando existe limitación de glutamato, la expresión de *GLT1* se regula por Gln3p, Gcn4p y posiblemente por un factor de la familia Cys_6-Zn_2. Los reguladores Gln3p y Gcn4p pertenecen a dos sistemas distintos de control general: al sistema de represión catabólica nitrogenada (NCR) y al control general de aminoácidos (GCN), respectivamente. El control específico de la expresión de *GLT1* posiblemente está mediado por un activador

de la familia Cys_6-Zn_2, y por el sistema de represión que responde a la concentración de glutamato intracelular. Los resultados sobre la regulación de *GLT1* sugieren un papel importante de GOGAT en la reasimilación del amonio que proviene de la degradación de fuentes secundarias de nitrógeno, en condiciones de limitación de glutamato, y en general, cuando hay un desbalance en la poza de aminoácidos.

El análisis de mutantes estructurales que no presentan actividad de $NADP^+$-GDH1 o de GOGAT, permitió concluir que *S. cerevisiae* utiliza principalmente a la enzima $NADP^+$-GDH1 cuando el amonio es la única fuente de nitrógeno. Dobles mutantes que carecen de ambas enzimas no son auxótrofas de glutamato; este resultado sugirió una vía alternativa de biosíntesis de este compuesto. El análisis de la secuencia del genoma completo de levadura, confirmó la presencia del gen *GDH3,* el cual codifica para una enzima similar a la proteína $NADP^+$-GDH1. La interrupción de los genes *GLT1*, *GDH1* y *GDH3*, resulta en una cepa auxótrofa de glutamato.

En condiciones aerobias, el metabolismo de la glucosa en *S. cerevisiae* es principalmente fermentativo, mientras que el de la levadura *K. lactis* es en su mayor parte oxidativo. En el presente trabajo se demuestra que *K. lactis* utiliza a la enzima $NADP^+$-GDH y al ciclo GS-GOGAT para asimilar amonio y biosintetizar glutamato. Posiblemente el ciclo GS-GOGAT es el principal responsable de la biosíntesis de glutamato en amonio como fuente de nitrógeno, en contraste con los resultados obtenidos en *S. cerevisiae*. Estos estudios permiten iniciar un análisis comparativo de las vías de asimilación de amonio y biosíntesis de glutamato en estos organismos.

ABSTRACT

Ammonium assimilation and glutamate biosynthesis in the yeast *S. cerevisiae* is carried out by the glutamate dehydrogenase ($NADP^+$-GDH1) enzyme and the concerted action of glutamine synthetase and glutamate synthase (GS-GOGAT pathway). In this work we present the characterization of this metabolic pathways and the description of an enzyme that achieves the same function: the $NADP^+$-GDH3 protein.

GOGAT biochemical characterization, cloning of *GLT1* gene which codifies for this enzyme, and construction of *glt1* structural mutants, determined the presence of a unique enzyme with GOGAT activity in this yeast. Comparison of the amino acid sequence of this enzyme with the sequence reported for GOGAT enzyme from other organisms, showed that functional domains and structural organization of this protein is highly conserved, and that it has more similarity to the eukaryotic enzyme.

Cellular levels of GOGAT enzyme from *S. cerevisiae* are regulated at the transcriptional level by the nitrogen source. *GLT1* gene expression is negatively regulated by glutamate, the GOGAT reaction product. Under glutamate excess conditions, *GLT1* expression is governed by both glutamate mediated-repression and activation mediated by Gln3p and Gcn4p. In conditions of glutamate limitation, *GLT1* expression is regulated by Gln3p, Gcn4p and possibly by a Cys_6-Zn_2 family factor. Gln3p and Gcn4p regulators belong to different general control systems: the nitrogen catabolic repression system (NCR) and the nitrogen control system (GCN), respectively. Specific control of *GLT1* expression possibly is mediated by the activator of the Cys_2-Zn_6 family, and by the repression system that responds to the intracellular glutamate concentration. *GLT1* regulation results suggest an important role for GOGAT in ammonium assimilation that comes from

secondary nitrogen sources degradation, under glutamate limiting conditions, and in general, when there is an imbalance in the amino acid pool.

The analysis of structural mutants which are impaired in their $NADP^+$-GDH1 or GOGAT activity, allowed to conclude that *S. cerevisiae* mainly uses the $NADP^+$-GDH1 pathway in ammonium as the sole nitrogen source. Double mutants lacking both metabolic routes are not glutamate auxotrophs; this result suggested the existence of an alternative route. Sequence analysis of the yeast genome, confirmed the presence of *GDH3* gene, which codifies for an enzyme similar to $NADP^+$-GDH1. A glutamate auxotroph results from the *GLT1*, *GDH1* and *GDH3* gene disruption.

Under aerobic conditions, glucose metabolism in *S. cerevisiae* is mainly fermentative, while in *K. lactis* is mainly oxidative. The present work demonstrates that *K. lactis* uses the $NADP^+$-GDH and the GS-GOGAT pathways for ammonium assimilation and glutamate biosynthesis. GS-GOGAT pathway possibly is the main pathway for glutamate biosynthesis in ammonium as the nitrogen source, as opposed of the results found in *S. cerevisiae*. These studies enable to initiate a comparative analysis of the ammonium assimilation and glutamate biosynthesis pathways in these organisms.

Capítulo I

INTRODUCCION GENERAL

1. Vías de asimilación de amonio y biosíntesis de glutamato.

Los organismos vivos tienen la capacidad de incorporar el nitrógeno que proviene del amonio para la biosíntesis de los compuestos nitrogenados. El glutamato y la glutamina son los productos de la vía de asimilación de amonio de la célula, y los donadores de nitrógeno para la biosíntesis de aminoácidos, purinas, pirimidinas, aminoazúcares y otras moléculas nitrogenadas.

Se han descrito dos rutas metabólicas que llevan a cabo la asimilación de amonio y la biosíntesis de glutamato (Fig. 1): el ciclo formado por la acción conjunta de las enzimas glutamino sintetasa (GS) y glutamato sintasa (GOGAT); y la enzima glutamato deshidrogenasa (GDH). En el ciclo GS-GOGAT, la enzima GS cataliza la aminación del glutamato para formar glutamina, requiriendo una molécula de ATP; después el grupo amido de la glutamina se transfiere al 2-oxoglutarato mediante la acción de GOGAT, lo que resulta en la síntesis neta de una molécula de glutamato a partir de 2-oxoglutarato y amonio (Tempest y cols., 1970). La enzima GDH asimila amonio y sintetiza glutamato a partir de amonio y 2-oxoglutarato (Holzer y Schneider, 1957). El cofactor en la reacción es el $NADP^+$.

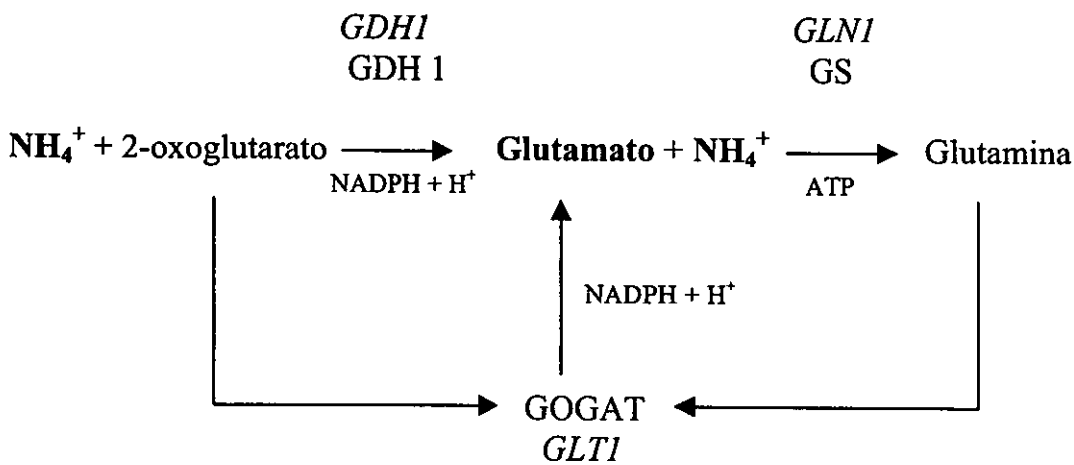

Fig. 1. Vías de asimilación de amonio y biosíntesis de glutamato en *Saccharomyces cerevisiae*.

La enzima GOGAT (glutamina (amida): 2-oxoglutarato amidotransferasa oxidorreductasa) fue descrita por primera vez en *Klebsiella aerogenes*, por el grupo de Tempest (1970). Desde entonces se ha encontrado que participa en la asimilación de amonio y en la biosíntesis de glutamato en una variedad de microorganismos, entre ellos bacterias y hongos (Tempest y cols., 1970; Senior, 1975; Hummelt y Mora, 1980; Bravo y Mora, 1988; Marqués y cols., 1992), así como en plantas superiores (Anderson y cols., 1989; Sakakibara y cols., 1991). La enzima GDH se encuentra en microorganismos, plantas y animales. Algunas especies de bacterias no tienen actividad de GDH, mientras que los animales superiores generalmente no tienen actividad de GOGAT. El papel fisiológico de la vía GDH y el ciclo GS-GOGAT parece depender de las condiciones en las cuales están creciendo los organismos. Por un lado influyen los parámetros cinéticos de las enzimas GDH, GS y GOGAT; y por otro, la regulación de las actividades enzimáticas y la diferencia en el patrón de expresión transcripcional de los genes que las codifican (Helling y cols., 1994).

En *Escherichia coli*, se ha propuesto que el ciclo GS-GOGAT puede tener por lo menos dos funciones, que no pueden ser sustituidas por la vía GDH: la primera es que GS-GOGAT asimila el amonio en moléculas orgánicas cuando la concentración externa de amonio es baja y la célula no está en condiciones de limitación de energía (Reitzer y Magazanik, 1987; Helling, 1994; Saroja y Gowrishankar, 1996); y la segunda es que regula los niveles intracelulares de glutamina (Reitzer y Magazanik, 1987). La afinidad por amonio de la enzima GDH es baja (Km 3 mM), y la reacción que cataliza se desplaza hacia la degradación de glutamato cuando la concentración de amonio es limitante (Miller y Stadtman, 1972). Mientras que la afinidad de GS por amonio es alta (Km

0.2 mM) y su reacción es prácticamente irreversible (Denton y Ginsburg, 1970). El ciclo GS-GOGAT en condiciones de escasez de amonio se encarga de asimilar este compuesto y sintetizar glutamato; sin embargo, la vía GS-GOGAT es más costosa que la vía GDH porque consume una molécula de ATP en cada ciclo.

El trabajo reciente de Helling (1994) en *E. coli*, sugiere que la vía GDH tiene un papel importante en la síntesis de glutamato, en condiciones de exceso de amonio y limitación de energía (y de carbono), al tener un costo energético menor al del ciclo GS-GOGAT. Por otro lado, el ciclo GS-GOGAT es responsable de la síntesis de glutamato cuando el contenido energético es alto o cuando la concentración de amonio es limitante. Los experimentos de Saroja y Gowrishankar (1996) demostraron que la actividad de GDH aumenta en condiciones de limitación de carbono y nitrógeno, y en condiciones de estrés osmótico -donde la concentración intracelular de glutamato se eleva como mecanismo de protección; Csonka, 1989. A partir de estos resultados, proponen un papel importante de GDH durante el crecimiento y sobrevivencia de *E. coli* en su hábitat natural (intestino grueso de mamíferos, o bien en suelos, sedimentos o agua), donde existe limitación de carbono y de nitrógeno, y posiblemente estrés por desecación. Estos autores extienden la conclusión de Helling, sugiriendo que *E. coli* puede asimilar eficientemente el amonio por la vía GDH en condiciones de estrés osmótico, en condiciones de exceso de amonio y limitación de carbono, o bien, si está limitada simultáneamente de carbono y nitrógeno.

En plantas, el ciclo GS-GOGAT es la ruta principal de asimilación de amonio y síntesis de glutamato (Robertson y cols., 1975; Miflin y Lea, 1977). En plantas leguminosas se han descrito dos proteínas con actividad de GOGAT: la enzima Fd-GOGAT, que se encuentra en los cloroplastos y asimila el amonio que se produce por la reducción de

nitratos dependiente de luz, y el que proviene de la fotorrespiración; y la enzima NADH-GOGAT que se encuentra principalmente en tejidos no-verdes como raíces, brotes y nódulos. La actividad de la enzima NADH-GOGAT aumenta durante el desarrollo de los nódulos y está involucrada en la asimilación del amonio que proviene de la fijación bacteriana, por lo que se le ha identificado como una nodulina (Gregerson, 1993).

A diferencia de lo que ocurre en bacterias, donde la dirección de la reacción hacia la síntesis o la degradación de glutamato depende de la actividad de una sola enzima GDH, en hongos ambas actividades se llevan a cabo de manera independiente. La enzima biosintética utiliza $NADP^+$ como cofactor y está codificada por el gen *GDH1*; la enzima catabólica utiliza NAD^+ como cofactor y se encuentra codificada por el gen *GDH2*. El ciclo GS-GOGAT se ha considerado poco importante para la asimilación de amonio en las levaduras *Saccharomyces cerevisiae*, *Candida utilis* y *Candida boidinii*, donde la principal vía de asimilación tanto en exceso como en limitación de amonio es la vía $NADP^+$-GDH1. Sin embargo, en *Aspergillus nidulans*, *Neurospora crassa* y *Schizosaccharomyces pombe*, el ciclo GS-GOGAT se encarga de asimilar el amonio y sintetizar glutamato cuando el amonio se encuentra en concentraciones limitantes. El ciclo GS-GOGAT también es la principal vía de asimilación de amonio en varias especies patogénicas de *Candida spp*. En el hongo *N. crassa*, se ha sugerido que GOGAT tiene una función importante en la degradación de glutamina, al reciclar el nitrógeno orgánico de la glutamina al glutamato (Para una revisión ver Valenzuela, 1994).

2. Características de la enzima glutamato sintasa.

La enzima GOGAT es una flavoproteína que tiene centros fierro-azufre. Durante la reacción, la enzima transfiere reductivamente el grupo amido de la glutamina al 2-oxoglutarato, dando como resultado la síntesis de dos moléculas de glutamato. El donador de electrones de la reacción que llevan a cabo las distintas enzimas GOGAT, puede ser el NADPH, el NADH o la ferredoxina. En las enzimas GOGAT de *E. coli* y *A. brasilense,* el NADPH dona sus electrones a una flavina presente en el primer sitio activo, y éstos se transfieren a la flavina del segundo sitio activo, a través de los centros fierro-azufre. En el segundo sitio activo ocurre la reducción del iminoglutarato, formado a partir del 2-oxoglutarato y el amonio que proviene del grupo amido de la glutamina. El mecanismo cinético es del tipo Uni Uni Bi Bi ping-pong, en el cual la enzima alternativamente se reduce por el NADPH y se oxida por el iminoglutarato (Rendina y Orme-Johnson, 1978; Vanoni y cols., 1991).

La enzima bacteriana tiene una subunidad grande de 160 kD y una subunidad chica de 60 kD; en contraste con la enzima de organismos eucariontes, que se encuentra formada por monómeros de alrededor de 200 kD. La región que corresponde a la subunidad grande de la enzima NADPH-GOGAT de *E. coli* está conservada en las enzimas NADH-GOGAT y Fd-GOGAT de plantas; mientras que la región correspondiente a la subunidad pequeña solo se encuentra en la enzima NADH-GOGAT. Aparentemente la subunidad pequeña de la enzima GOGAT de bacterias y el dominio carboxilo terminal de la enzima NADH-GOGAT de plantas forman parte del sitio de unión al cofactor NADH o NADPH; esta región no se requiere cuando el cofactor es ferredoxina (Gregerson, 1993).

El sitio de unión a glutamina de las enzimas GOGAT comparte características con el dominio de unión a glutamina de las enzimas glutamina amidotransferasas de la subfamilia Ntn hidrolasas (antes denominada tipo-F o clase II). En las enzimas Ntn hidrolasas, el dominio de unión a glutamina se encuentra en la región amino-terminal de la proteína y contiene nueve residuos conservados. El primer aminoácido (Cys, Ser o Thr) tiene como función la transferencia del grupo amido de la glutamina. En la enzima glutamina PRPP amidotransferasa (glutamina fosforribosilpirofosfato amidotransferasa), que pertenece a esta familia y que al igual que la enzima GOGAT tiene una Cys en la posición 1 (Cys^1), se demostró que la cadena lateral de la Cys^1 forma un tiolato con la glutamina. El α-NH_2 de la Cys^1 sirve como aceptor de protones durante la formación del tiolato y como donador de protones para la transferencia del nitrógeno del grupo amido. Basados en la estructura obtenida por estudios de difracción de rayos X de las enzimas PRPP cristalizadas de *B. subtilis* y de *E. coli*, se ha encontrado que los aminoácidos importantes en la reacción catalítica están conservados en todas las enzimas Ntn hidrolasas; además de estos resultados, las interacciones que ocurren con el ligando en las enzimas Ntn hidrolasas, apoyan la idea de que estas proteínas son enzimas homólogas (Kim y cols., 1996).

Las enzimas glutamina PRPP amidotransferasas se dividen en dos grupos (Kim y cols., 1996): enzimas que contienen una secuencia propeptídica antes de la Cys^1 cuyo representante es la enzima de *B. subtilis*; y enzimas que no tienen este propéptido y que son similares a la enzima de *E. coli*. En este último grupo, la metionina previa a la Cys^1 de la enzima sin madurar, se elimina por la acción de una enzima metionina aminopeptidasa. A este respecto, todas las enzimas GOGAT secuenciadas hasta la fecha,

contienen una secuencia propeptídica que se corta para dar origen a la enzima madura (Gregerson y cols., 1993).

3. Control de la expresión de los genes que codifican para la enzima GOGAT.

A la fecha no se conoce cómo es la regulación transcripcional de los genes que codifican para la enzima GOGAT en organismos eucariontes. Los estudios que involucran el control de la expresión de estos genes se han realizado en procariontes, y se describen a continuación.

Escherichia coli. En *E. coli*, el operón *gltBDF* incluye los genes que codifican para la subunidad grande (*gltB*) y chica (*gltD*) de la enzima GOGAT, y un gen regulador (*gltF*) (Castaño y cols, 1988). El producto del gen *gltF* está involucrado en la represión transcripcional del operón *gltBDF* mediada por glutamato (Castaño y cols, 1992).

La proteína Lrp, controla la transcripción de más de 40 genes y operones en *E. coli*. Los miembros del regulón de Lrp incluyen genes y operones involucrados en la biosíntesis y degradación de aminoácidos, transporte de nutrientes y formación del pili. Lrp es una proteína dimérica con un dominio de unión a DNA formado por una estructura hélice-vuelta-hélice. Los estudios realizados por Wiese y cols. (1997) sugieren que Lrp puede doblar la cadena de DNA hacia sí misma. La proteína Lrp puede actuar como activador o como represor transcripcional, y la leucina, su co-regulador, puede o no ser requerida para inhibir o activar estas funciones (Wiese y cols., 1997).

La activación de la transcripción del operón *gltBDF* está mediada por Lrp. En el promotor de *gltBDF*, se encontró una secuencia palindrómica con alta afinidad por este regulador, flanqueada por dos secuencias de menor afinidad. Las secuencias están

ubicadas en la región entre –140 y –260 pb, río arriba del inicio de transcripción y relativamente alejadas de las secuencias que posicionan a la enzima RNAP. Las tres secuencias están localizadas en la misma cara de la hélice de DNA, lo que sugiere que las proteínas Lrp se unen al DNA cooperativamente: primero un dímero de Lrp ocupa la secuencia central y después se unen otros dos dímeros a las secuencias menos afines ayudados por interacciones proteína-proteína. Se ensambla así un complejo nucleoprotéico que altera la estructura del DNA, posiblemente mediante la formación de un asa, y que acerca al complejo y a la enzima RNAP permitiéndo la interacción entre ambos (Wiese y cols., 1997).

La leucina tiene un efecto mínimo sobre la actividad de Lrp en el promotor de *gltBDF*: reprime 2.2 veces su expresión. La presencia de leucina posiblemente causa un cambio conformacional en la proteína Lrp. Como consecuencia, este regulador no se une a la secuencia menos afín, localizada cerca del inicio de transcripción (Wiese y cols., 1997).

Bacillus subtilis. En *B. subtilis*, la regulación de la actividad de GOGAT ocurre a nivel transcripcional en función de la fuente de nitrógeno presente. Los niveles de transcripción de los genes *gltA* y *gltB*, se reprimen al crecer las células en glutamato, y aumentan en presencia de amonio. En glutamina, los niveles de transcripción son intermedios (Bohannon y cols., 1985).

La regulación dependiente de la fuente de nitrógeno, requiere de un factor transcripcional codificado por el gen *gltC*, el cual actúa positivamente en la expresión de *gltA* y *gltB*. La proteína GltC pertenece a la familia LysR de reguladores de transcripción bacterianos. Los miembros de esta familia generalmente activan la transcripción de sus genes blanco, los cuales se encuentran frecuentemente colocados junto al gen regulador y son

transcritos divergentemente. GltC se encuentra codificado río arriba y en dirección opuesta a los genes *gltA* y *gltB*. Además de estimular la transcripción de *gltAB* en condiciones de limitación de glutamato, GltC reprime la transcripción de su propio gen (Bohannon y Sonenshein, 1989).

Los inicios de transcripción de *gltA* y *gltC* están separados por 51-52 pares de bases. GltC se une, en esta región, a dos secuencias denominadas caja I y caja II, las cuales son similares a secuencias reconocidas por proteínas tipo LysR. La caja I se localiza en la posición –64 respecto al inicio de transcripción de *gltA* y la caja II en la región –35 del promotor de *gltA* (Belitsky y cols., 1995). El modelo de regulación de *gltA* y *gltC* descrito por Belitsky y cols. (1995) se describe a continuación. La proteína GltC se une a la caja I en todas las condiciones de crecimiento, y provoca que la transcripción de *gltC* se reprima alrededor de 10 veces. Cuando el ambiente intracelular indica que hay limitación de glutamato, GltC se une también a la caja II; esta unión se facilita por interacciones con las moléculas de GltC que se encuentran unidas a la caja I. Las moléculas de GltC que unen a la caja II interaccionan con la RNA polimerasa, estimulando la transcripción de *gltA*. Debido a que cada caja está formada por un palíndromo, posiblemente cada una de ellas une a un dímero de GltC y la regulación positiva de *gltA* depende de las interacciones dímero-dímero. Si el modelo es correcto, la regulación ocasionada por la fuente de nitrógeno podría reflejar los efectos de metabolitos en esta interacción dímero-dímero. La identidad de los metabolitos, que podrían informar sobre la disponibilidad de glutamato dentro de la célula, no se conoce.

Deshpande y cols. (1981) sugirieron que la glutamina, un substrato en la reacción que cataliza GOGAT, es el metabolito cuya concentración intracelular dirige de manera más

directa, la síntesis de la enzima GOGAT de *B. subtilis*. Ellos reportan que en los cultivos de células que crecen en glutamato más amonio (alta actividad de GOGAT), que se cambian a un medio únicamente con glutamato (baja actividad de GOGAT), ocurre un aumento tanto en la actividad de GS como en la poza intracelular de glutamina (8 veces). Las concentraciones de glutamato, aspartato, alanina y amonio no cambian. Después de que la concentración de glutamina alcanza su máximo, la actividad de GOGAT comienza a disminuir llegando a ser 2.5 veces menor que en glutamato más amonio. Por lo tanto, existe una relación inversa entre la actividad específica de GOGAT y la poza intracelular de glutamina.

Klebsiella aerogenes. En *K. aerogenes*, cuando el amonio está ausente, la síntesis de enzimas necesarias para convertir fuentes pobres de nitrógeno en amonio o glutamato aumenta, y la síntesis de las enzimas responsables de la asimilación de amonio en glutamato (GDH y GOGAT) se reprime. La regulación por nitrógeno de varios operones requiere del sistema NTR (sistema global de control por nitrógeno) y de la proteína NAC, la cual pertenece a la familia de reguladores LysR. La proteína NAC se sintetiza en respuesta a condiciones de limitación de amonio a través del sistema NTR; por un lado activa genes como hut (histidasa), put (prolina oxidasa) y ure (ureasa), y por otro reprime la expresión de *gdh* (GDH) y *gltBD* (GOGAT). El regulador NAC parece proveer un medio para acoplar la expresión de algunos genes que son dependientes de sigma-70, al sistema NTR que depende de sigma-54 (Macaluso y cols., 1990; Bender, 1991).

En medio rico (LB) con glutamina como fuente de nitrógeno, la actividad de GDH y GOGAT se reprimen, posiblemente por la presencia de glutamato en este medio; sin

embargo, esta represión no está mediada por NAC. Por lo tanto, debe existir otro sistema de regulación de estas dos enzimas en *K. aerogenes*. (Bender y cols., 1983)

4. Generalidades sobre el control transcripcional en *S. cerevisiae.*

El complejo de inicio de síntesis de RNA mensajero en eucariontes está formado por la holoenzima (formada por la enzima RNA polimerasa II asociada al mediador) y por los factores generales TFIIs (TFII-A, TFII-B, TFII-D, TFII-F, TFII-E y TFII-H). El factor general TFII-D está formado por las proteínas TAFs y la proteína TBP, ésta última reconoce una secuencia específica en el promotor denominada caja TATA. El ensamble del complejo de inicio de transcripción comienza al unirse TBP a la caja TATA, seguido de los TFIIs y de la holoenzima (Roeder, 1996). En algunos promotores eucariontes existe una secuencia, denominada INR, dentro de la cual ocurre el inicio de transcripción (McNeil y Smith, 1985).

En los promotores de *S. cerevisiae* la caja TATA se puede localizar en la región comprendida entre –40 y –120 nucleótidos respecto al sitio de inicio de transcripción (el cual define la posición +1). La secuencia consenso de la caja TATA es 5'-TATAAA-3' (Harbury y Struhl, 1989; Iyer y Struhl, 1995a); mientras que el elemento INR presenta dos consensos: 5'-TCGA-3' y 5'-PuPuPyPuPu-3' (Hahn y cols., 1985).

Los reguladores de la transcripción en *S. cerevisiae* se unen a secuencias de activación (UASs) o de represión (URSs) que pueden estar a cientos o miles de bases alejados de la caja TATA y del inicio de transcripción. Se ha propuesto que los activadores y represores que se unen a estas secuencias actúan tanto en el reclutamiento de TBP a la caja TATA, como en el reclutamiento de la holoenzima (Stargell y Struhl, 1996). La capacidad que

tienen las proteínas reguladoras de funcionar a distancia requiere de proteínas adaptadoras (coactivadores o correpresores) que no se unen al DNA, y que sirven de enlace entre los reguladores unidos a las secuencias UAS o URS y la maquinaria transcripcional ensamblada sobre la caja TATA (Guarente, 1992). Algunos de estos coactivadores, como el complejo ADA, el complejo SAGA y otros complejos como el SWI-SNF o el RSC, se encargan de remodelar la estructura de la cromatina, y se ha sugerido que facilitan: 1) la unión de los reguladores a secuencias específicas en el DNA, 2) el posicionamiento tanto de TBP como del resto de la maquinaria transcripcional sobre la caja TATA y el sitio de inicio de transcripción, y 3) el proceso de elongación (Cairns y cols., 1996; Stargell y Struhl, 1996; Grant y cols., 1997; Pérez-Martín y Johnson, 1998).

Muchos promotores de levadura contienen secuencias homopoliméricas de timinas, poli(dA-dT), que se requieren para mantener niveles normales de expresión génica (Struhl, 1985). Estas secuencias forman una hélice con un número menor de nucleótidos (10 pb en lugar de 10.6 pb), tienen un surco menor más estrecho y son estructuralmente rígidas. Cuando estas secuencias son perfectas (solo timinas), su función parece depender de su estructura intrínseca y no de la interacción con proteínas que unen secuencias específicas en el DNA; de hecho, la presencia de secuencias poli(dA-dT) altera localmente la estructura de la cromatina de manera que el DNA adyacente a esta secuencia es más accesible a proteínas que cortan o modifican el DNA. Esta perturbación de la cromatina se extiende a una región de aproximadamente 100 pb a cada lado de la secuencia poli(dA-dT), una longitud que es solo un poco mayor a la región ocupada por un nucleosoma (Iyer y Struhl, 1995b). Iyer y Struhl (1995b) han propuesto que la secuencia poli(dA-dT) aumenta la accesibilidad del DNA hacia los factores

transcripcionales que se unen a secuencias que están cercanas, posiblemente porque su estructura rígida desestabiliza o altera la conformación del nucleosoma donde se encuentran. Cuando las secuencias son imperfectas, es posible que sus efectos transcripcionales sean mediados en parte o completamente por proteínas que unen al DNA de manera específica (Lue y cols., 1989). Por otro lado, existe una proteína represora, la datina, que es capaz de unirse a las regiones poli(dA-dT) perfectas de más de 10 pb, cuya acción represora ocurre al ocupar las secuencias poli(dA-dT) e impedir su función (Iyer y Struhl, 1995b).

En general las proteínas reguladoras presentan tres actividades: unión a secuencias específicas en el DNA, activación o represión de la transcripción, y respuesta a señales reguladoras. Los dominios de unión a DNA de las proteínas reguladoras pueden contener tres tipos de motivos predominantes. El primero es un motivo "hélice-vuelta-hélice", también conocido como homeodominio. Un segundo motivo es el "dedo de zinc", donde un átomo de zinc se coordina por un par de cisteínas y un par de histidinas (Cys_2-His_2), o bien por cuatro cisteínas (Cys_4). A este último grupo pertenecen los miembros de la familia GATA de *S. cerevisiae*: Gln3p, Gat1p, Dal80p y Deh1p (Coffman y cols., 1997). Una variante de este motivo es el "grupo con dos núcleos de zinc Cys_6-Zn_2" que se encuentra en un gran número de proteínas reguladoras de hongos. En *S. cerevisiae* se han encontrado 56 proteínas que comparten este dominio, de las cuales solo se conoce la función de 14 (Schjerling y Holmberg, 1996). Estas proteínas reconocen elementos CGG organizados como: palíndromos (5'-CGG-N_X-CCG-3'), repetidos directos (5'-CGG-N_X-CGG-3') y palíndromos invertidos (5'-CCG-N_X-CGG-3') (Hellauer y cols., 1996). El tercer motivo es la "cremallera o cierre de leucinas". Este motivo se encuentra en el

dominio de unión al DNA del activador Gcn4p de levadura, el cual se une al DNA como dímero (Sellers y Struhl, 1989).

5. Regulación transcripcional de los genes involucrados en la biosíntesis de aminoácidos en *S. cerevisiae.*

Los genes que codifican para las enzimas que biosintetizan aminoácidos en *S. cerevisiae*, están regulados por una interacción compleja entre numerosos mecanismos de control [Hinnebusch (1992) ha hecho una revisión de estos mecanismos]. La mayor parte de estos genes están regulados por el control general de aminoácidos (GCN), a través de la acción de Gcn4p. Este factor transcripcional reconoce la secuencia AP1 (5'-TGACTC-3') y secuencias relacionadas (Hinnebusch y Finck, 1983; Mavrothalassitis y cols., 1990; Tavernarakis y Thireos, 1997).

Se ha determinado que en presencia de una señal de limitación de aminoácidos, aumentan los niveles de traducción del RNAm que codifica para el activador Gcn4p; el aumento en la cantidad de proteína Gcn4 permite a su vez un incremento, de dos a diez veces, en la expresión de los genes biosintéticos de aminoácidos (Hinnebusch, 1986). Para alcanzar estos niveles máximos de expresión dependiente de Gcn4p, algunos genes requieren de la acción de proteínas reguladoras globales o del coactivador ADA. Entre los reguladores globales se incluyen Rap1p y Abf1p, los cuales, al igual que el complejo ADA, posiblemente alteran la estructura de la cromatina (Devlin y cols., 1991; Georgakopoulos y Thireos, 1992; Martens y Brandl, 1994). En ausencia de una señal de limitación de aminoácidos, como ocurre durante el crecimiento en medio mínimo, algunos genes requieren de la acción de Gcn4p, o de proteínas activadoras específicas, para mantener

una expresión eficiente (Arndt y cols., 1987; Tice-Baldwin y cols., 1989; Hinnebusch, 1992). Por otro lado, se ha reportado que las regiones poli(dA-dT) presentes en el promotor del gen *HIS3*, se requieren para alcanzar los niveles normales de expresión mediados por Gcn4p, tanto en medio mínimo como en condiciones donde existe limitación de aminoácidos (Struhl, 1982; Struhl, 1985).

Además de los mecanismos de activación, muchos genes biosintéticos de aminoácidos están sujetos a un control específico de represión, que reduce la expresión de los genes cuando el aminoácido que constituye el producto final de la vía está presente en el medio (Hinnebusch, 1992). A este respecto, se ha reportado que los factores de regulación global también pueden funcionar como represores en los genes regulados por Gcn4p (Kunzler y cols., 1995).

6. Control transcripcional de los genes del catabolismo nitrogenado en *S. cerevisiae*.

S. cerevisiae puede utilizar una gran variedad de compuestos como fuentes de nitrógeno. Ciertos compuestos nitrogenados (asparagina, glutamina y amonio en algunas cepas) se utilizan de manera preferente. Cuando estas fuentes primarias de nitrógeno no están disponibles, o están presentes en concentraciones suficientemente bajas para limitar el crecimiento, se utilizan otras fuentes de nitrógeno (purinas, pirimidinas, amidas o aminoácidos). La transcripción de los genes estructurales que codifican para las enzimas y permeasas que permiten utilizar estas fuentes secundarias, se reprime en presencia de las fuentes primarias de nitrógeno. A este efecto se le denominó represión catabólica nitrogenada (NCR) (Cooper, 1982). Recientemente, Magasanik (1992) propuso utilizar el

término regulación por nitrógeno (NR), el cual es más correcto al no involucrar el catabolismo de las fuentes primarias de nitrógeno en la regulación.

La activación transcripcional de la mayoría de los genes regulados por nitrógeno requiere de la presencia de dos señales positivas distintas: una señal global indicando ausencia de una fuente primaria de nitrógeno (desrepresión catabólica nitrogenada), y una señal específica para cada vía catabólica, que informa sobre la presencia de un sustrato o un intermediario de esa vía (inducción). La inducción requiere de reguladores exclusivos de cada vía; algunos de estos reguladores pertenecen a la familia de proteínas con dominios Cys_6-Zn_2. Algunos genes están controlados solo por represión/desrepresión catabólica nitrogenada, y no requieren de inducción (ver revisión de Marzluf, 1997).

Los reguladores globales Gln3p y Gat1p, son los mediadores de la represión /desrepresión catabólica nitrogenada: en ausencia de una fuente primaria de nitrógeno activan la transcripción de los genes del catabolismo nitrogenado. Estos reguladores presentan un motivo "dedo de zinc" en su dominio de unión al DNA, y reconocen secuencias GATA (5'-GATAAG-3'), o elementos UAS_{NTR}, presentes en las regiones promotoras (Bysani y cols., 1991; Coffman y cols., 1997). La expresión del gen *GLN3* es constitutiva, por lo que se ha sugerido que la actividad de Gln3p, en presencia de una fuente primaria de nitrógeno, se regula principalmente impidiendo su unión al DNA, o afectando su capacidad de llevar a cabo la activación transcripcional (Minehart y Magasanik, 1991). Esta regulación parece involucrar la participación de Ure2p y de una o más proteínas que no se han caracterizado (Coffman y cols. 1994; Coffman y cols., 1997). El mecanismo por el cual Ure2p afecta la función de Gln3p no se conoce. Por un lado, Ure2p presenta similitud a enzimas glutatión-S-transferasas, lo cual sugiere que su

función bioquímica podría ser la modificación post-traduccional de Gln3p (Coshigano y Magasanik, 1991). Por otro lado, los resultados de Blinder y cols. (1996) sugieren que Ure2p se une directamente a Gln3p.

Los reguladores Dal80p y Dehlp presentan un motivo "dedo de zinc" y un motivo "cremallera de leucina" en su dominio de unión a DNA. Estos reguladores reprimen la transcripción de genes del catabolismo nitrogenado al unirse a secuencias GATA (URS_{GATA}). La secuencia óptima a la cual se une Dal80p, consiste de un par de elementos GATA, separados entre sí por 15 a 35 nucleótidos. Estos elementos pueden estar colocados en forma palindrómica (5'-CTTATC-N_{15-35}-GATAAG-3') o bien como repetidos directos (5'-GATAAG-N_{15-35}-GATAAG-3') (Cunningham y Cooper, 1993; Coffman y cols., 1997).

La expresión de los genes del catabolismo nitrogenado involucra la participación de los cuatro factores de la familia GATA; al unirse a secuencias relacionadas, posiblemente los reguladores antagonizan la operación de unos y otros (Cunningham y cols., 1994). Se ha sugerido que el número y orientación de los elementos GATA determinan la especificidad de unión de las distintas proteínas de la familia GATA a los promotores (Coffman y cols., 1997). De acuerdo con ésta hipótesis, la expresión de algunos genes depende de ambos activadores Gln3p y Gatlp, mientras que la expresión de otros genes depende de solo uno de los activadores. Así mismo, algunos genes se regulan por Dal80p y/o por Dehlp (Coffman y cols., 1997). Por otro lado, en algunos promotores, la activación dependiente de cierto regulador posiblemente está determinada por la fuente de nitrógeno presente: el gen que codifica para la permeasa general de aminoácidos *GAP1* aumenta su expresión en glutamato por la acción de Gln3p, mientras que en

amonio, urea o prolina el aumento es mediado por Gatlp (Stanbrough y Magazanik, 1996). Dehlp funciona principalmente en fuentes primarias de nitrógeno, en condiciones donde la expresión de Dal80p está severamente reprimida (Coffman y cols., 1997). En la figura 2 se muestra el circuito de activación y represión de los genes del catabolismo nitrogenado en *S. cerevisiae* propuesto por Coffman y cols. (1997).

La expresión de cada uno de los genes que codifican para los reguladores de la familia GATA, presenta un patrón distinto de regulación, que depende de la calidad de la fuente de nitrógeno disponible y de la presencia o ausencia de los otros tres reguladores (ver Fig. 2): la expresión del gen *GLN3* es constitutiva; la expresión de *GAT1* depende de Gln3p y está regulada por Dal80p; la expresión de *DAL80* depende de Gln3p y Gatlp y se autorregula por Dal80p; la expresión de *DEH1* es independiente de Gln3p, depende moderadamente de Gatlp y está fuertemente regulada por Dal80p (Coffman y cols., 1997). Coffman y cols. (1997) sugieren que esta regulación permite a las células responder finamente a una variedad de estímulos ambientales, permitiendo una transición lenta de una respuesta a otra.

Rai y cols. (1995) determinaron que los sitios UAS_{NTR} pueden funcionar en combinación con otros elementos *UAS* (si responden a inducción se les denomina *UIS*), originando patrones excepcionales de regulación por nitrógeno. Las respuestas reguladoras que se observan son un híbrido que consiste de características derivadas del sitio UAS_{NTR} así como del sitio no relacionado a UAS_{NTR}. En el promotor de *GLN1*, el sitio no relacionado a UAS_{NTR} es 5'-TTTGTTTAC-3', mientras que en el promotor de *PUT1* es el elemento inducible 5'-CGG-N_{10}-CCG-3' (*UIS*), el cual es reconocido por la proteína Put3, un miembro de la familia Cys_6-Zn_2. El activador Put3p se encuentra unido a las secuencias

UIS presentes en el gen *PUT1* tanto en presencia como en ausencia del inductor prolina. Cuando existe prolina en el medio, esta molécula le confiere a Put3p, de alguna manera, la capacidad de activar la expresión del gen *PUT1*. El elemento en *cis* de *GLN1* es incapaz de llevar a cabo la activación de la transcripción por sí mismo; este sitio y las regiones *UIS* parecen actuar aumentando el potencial de activación de los UAS_{NTR} adyacentes.

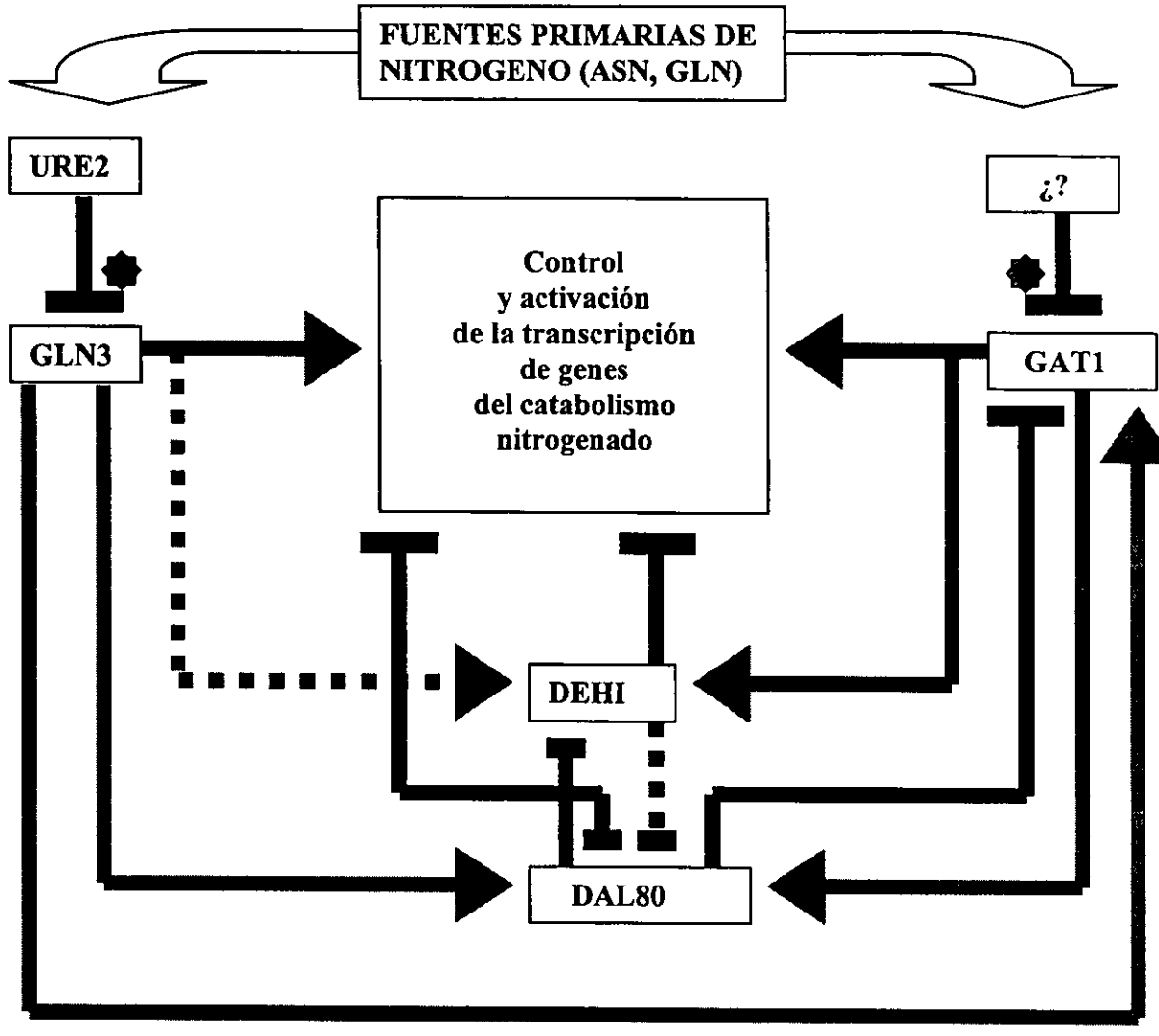

Fig. 2. Modelo que representa el circuito de regulación que lleva a cabo la activación y la represión de los genes del catabolismo nitrogenado en *S. cerevisiae*. Las flechas indican una regulación positiva; las barras representan una regulación negativa. El asterisco representa una regulación a nivel post-transcripcional. Obtenido de Coffman y cols.(1997).

7. Antecedentes y objetivos.

La vía $NADP^+$-GDH1 y el ciclo GS-GOGAT están presentes en la levadura *S. cerevisiae*. La existencia de dos vías que llevan a cabo la asimilación de amonio y biosíntesis de glutamato en esta levadura, ha llevado a especular sobre la posibilidad de que cada una tuviera un papel fisiológico diferente. El metabolismo celular podría requerir la expresión de una u otra vía, o bien, la función simultánea de ambas, dependiendo de las condiciones de crecimiento.

Para estudiar la función de cada una de estas rutas metabólicas se han obtenido mutantes que carecen tanto de la actividad de $NADP^+$-GDH1 (Drillien y Lacroute, 1972), como de la actividad de GOGAT (Folch y cols., 1989; Miller y Magazanik, 1990). Las cepas sin actividad de NADP+-GDH1 portan mutaciones en el gen estructural *GDH1* (Drillien y Lacroute, 1972; Folch y cols., 1989); sin embargo, las mutaciones que afectan la actividad de GOGAT se encuentran en genes reguladores (Guzmán, 1998; Folch y cols., 1989; Miller y Magazanik, 1990). La obtención de una cepa que porte una mutación en el gen estructural de GOGAT no ha podido lograrse por los métodos de mutagénesis al azar y selección de fenotipos. Para obtener estas cepas se podría realizar la mutagénesis dirigida del gen estructural que codifica para GOGAT.

Por otro lado, la obtención de una cepa doble mutante, que no presente actividad de GOGAT ni de $NADP^+$-GDH1, permitiría definir si existe una vía alternativa de asimilación de amonio y biosíntesis de glutamato en *S. cerevisiae*.

Nuestro grupo está realizando una labor conjunta para estudiar las vías de asimilación de amonio y de biosíntesis de glutamato de las levaduras *S. cerevisiae* y *K. lactis*. El trabajo de investigación desarrollado en esta tesis tiene como objetivo central el análisis de la función fisiológica de la enzima GOGAT de *S. cerevisiae*. Con este fin se llevaron a cabo los estudios que se describen a continuación, de los cuales el trabajo experimental a mi cargo quedaría descrito principalmente en los puntos 1, 2 y 3:

1. La caracterización bioquímica de la enzima GOGAT y la clonación del gen estructural (*GLT1*) que la codifica (Capítulo II).

2. El análisis estructural de la enzima GOGAT, definiendo los posibles dominios funcionales de esta enzima mediante el estudio de su secuencia de aminoácidos (Capítulo III).

3. El estudio de la regulación transcripcional del gen *GLT1* en respuesta a la fuente de nitrógeno presente; así como la determinación de los elementos en *cis* y los factores en *trans* que intervienen en esta regulación (Capítulo IV).

4. El análisis de la función fisiológica de la vía $NADP^+$-GDH1 y el ciclo GS-GOGAT, así como determinar si existe una vía alternativa de asimilación de amonio y biosíntesis de glutamato en *S. cerevisiae*, mediante la obtención y caracterización de cepas con mutaciones en los genes estructurales *GLT1*, *GDH1* y en ambos (Capítulo V).

La diferencia fisiológica principal entre *S. cerevisiae* y otras levaduras es su habilidad para fermentar eficientemente los azúcares bajo condiciones de anaerobiosis. Además esta levadura presenta una duplicación considerable de su genoma (50% aproximadamente). Wolfe y Shields (1997) sugieren que ocurrió una duplicación del genoma completo de *S. cerevisiae*, y que este evento fue muy importante en la evolución adaptativa al crecimiento anaerobio, manteniéndose finalmente solo una fracción del genoma duplicado. En contraste con *S. cerevisiae*, la levadura *K. lactis* utiliza un metabolismo principalmente oxidativo, es un aerobio estricto y los datos obtenidos a la fecha sugieren que su genoma se encuentra en copia única (Wésolowski-Louvel y cols. 1996; Wolfe y Shields, 1997). Los géneros *Saccharomyces* y *Kluyveromyces* son parientes cercanos desde el punto de vista evolutivo; sin embargo, los estudios que involucran la comparación de las secuencias de sus genes y el orden en el cual éstos se encuentran, sugieren que *S. cerevisiae* y *Kluyveromyces* divirgieron antes de que ocurriera la duplicación génica en *S. cerevisiae* (Wolfe y Shields, 1997).

5. El estudio comparativo de las vías de asimilación de amonio y de biosíntesis de glutamato que utilizan las levaduras *S. cerevisiae* y *K. lactis*, podría ayudar a entender la función fisiológica de cada una de estas rutas metabólicas. Con este fin nuestro grupo ha comenzado el análisis de las vías que utiliza la levadura *K. lactis* para asimilar amonio y biosintetizar glutamato; así como el estudio del posible papel fisiológico de estas vías, en cepas que presentan una interrupción del gen estructural que codifica para la enzima $NADP^+$-GDH (Capítulo VI).

Capítulo II

Saccharomyces cerevisiae has a single glutamate synthase gene coding for a plant-like high-molecular-weight polypeptide.

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Saccharomyces cerevisiae Has a Single Glutamate Synthase Gene Coding for a Plant-Like High-Molecular-Weight Polypeptide†

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Purification of the glutamate synthase (GOGAT) enzyme from *Saccharomyces cerevisiae* showed that it is an oligomeric enzyme composed of three identical 199-kDa subunits. The GOGAT structural gene was isolated by screening a yeast genomic library with a yeast PCR probe. This probe was obtained by amplification with degenerate oligonucleotides designed from conserved regions of known GOGAT genes. The derived amino-terminal sequence of the GOGAT gene was confirmed by direct amino-terminal sequence analysis of the purified protein of 199 kDa. Northern (RNA) analysis allowed the identification of an mRNA of about 7 or 8 kb. An internal fragment of the GOGAT gene was used to obtain null GOGAT mutants completely devoid of GOGAT activity. The results show that *S. cerevisiae* has a single NADH-GOGAT enzyme, consisting of three 199-kDa monomers, that differs from the one found in prokaryotic microorganisms but is similar to those found in other eukaryotic organisms such as alfalfa.

The existence of two pathways for glutamate biosynthesis has been demonstrated in several microorganisms (4, 16, 18, 31, 36, 40) and higher plants (1, 6, 15, 23, 32, 39). One pathway consists of the action of the NADP-dependent glutamate dehydrogenase, and the other involves the concerted action of glutamine synthetase and glutamate synthase (GOGAT).

Escherichia coli NADPH-GOGAT and *Azospirillum brasilense* NADPH-GOGAT have been extensively studied. They are composed of two subunits with molecular masses of 135 and 53 kDa (24, 29). The genes that code for the large and small polypeptides have been cloned and sequenced previously (9, 27, 28).

In higher plants, GOGAT occurs in three forms that differ in molecular mass, kinetics, location within the plant, and cofactor specificity (38). The genes that code for NADH-GOGAT from alfalfa (*Medicago sativa*) (14) and Fd-GOGAT from maize (*Zea mays*) (32) have also been cloned. Comparative analysis of the amino acid sequences of these two GOGATs with that of *E. coli* revealed highly conserved regions (14).

A protein which bears GOGAT activity has also been purified from baker's yeast. This protein is a dimer composed of a large subunit (169 kDa) and a small subunit (61 kDa) (19).

The isolation of *Saccharomyces cerevisiae* mutants impaired in GOGAT activity has previously been reported (7, 25). Genetic analysis of one of these mutants, CN39 (*MATα gdh gus1 gus2*), showed that its lack of GOGAT activity was due to the presence of two mutations (*gus1* and *gus2*), which suggests the existence of two GOGAT enzymes in *S. cerevisiae* (7).

In order to definitively establish whether there is one GOGAT enzyme or two GOGAT enzymes in *S. cerevisiae*, we decided to purify the enzyme and construct null GOGAT mutants.

We report here the purification of this enzyme and cloning of the yeast GOGAT structural gene. Our results show that the protein is a homotrimeric enzyme composed of 199-kDa polypeptides. We were also able to isolate GOGAT-disrupted yeast mutants completely devoid of NADH-GOGAT activity.

Our results show that *S. cerevisiae* has a single NADH-GOGAT enzyme composed of 199-kDa monomers similar to those found in other eukaryotic organisms.

MATERIALS AND METHODS

Purification of GOGAT from *S. cerevisiae*. *S. cerevisiae* CN36 (*MATa his lys1 gdh1 GUS1 GUS2*) (7) was grown in a 15-liter fermentor on 10 liters of minimal medium which contained salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco Laboratories, Detroit, Mich.), supplemented with 2% glucose and 40 mM $(NH_4)_2SO_4$. Histidine and lysine were added at 0.01% (wt/vol) as auxotrophic requirements. Cultures were incubated at 30°C and 300 rpm and aerated with 7 liters of oxygen per min. Cells were harvested at an optical density at 600 nm of 2.0 and stored at −70°C until used. GOGAT was purified by the following procedure.

(i) Step 1: crude extract. Cells were thawed and resuspended in 1 ml of extraction buffer (0.1 M potassium phosphate [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 μl of TLCK [Nα-*p*-tosyl-L-lysine chloromethyl ketone] per ml) per g of cells. Crude extracts were obtained after mechanical disruption of cells with a Bead-Beater (10 cycles of 1 min). After centrifugation at 35,000 × *g* for 30 min, protein extract was diluted to 30 mg/ml.

(ii) Step 2: ammonium sulfate precipitation. Proteins which precipitated at between 30 and 53% saturation with ammonium sulfate were resuspended in 20 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 2 mM 2-oxoglutarate, 1 mM DTT, and 1 mM PMSF (buffer A) at a concentration of 60 mg/ml, as described by Sakamoto et al. (33). The mixture was then dialyzed against 8 liters of buffer A without 2-oxoglutarate and DTT (buffer B).

(iii) Step 3: DEAE Bio-Gel A chromatography. The dialyzed fraction was applied to a DEAE Bio-Gel A column (23 by 2.8 cm) equilibrated with buffer A. After sample application, the column was washed with 4 column volumes of buffer A. GOGAT was subsequently eluted with a linear KCl gradient (0 to 0.5 M) (10 column volumes). Fractions with GOGAT activity were pooled and dialyzed against buffer B.

(iv) Step 4: phenyl-Sepharose chromatography. The fraction from the previous step was taken to 12% saturation with ammonium sulfate. This sample was applied to a phenyl-Sepharose column (23 by 1.7 cm) equilibrated with 12% saturated ammonium sulfate in buffer A in the absence of PMSF. The column was washed with equilibrating buffer and eluted with a linear gradient from 12 to 0% saturation in ammonium sulfate and from 0 to 5% ethylene glycol in buffer

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† This paper is dedicated to the memory of Aurora Brunner Liebshard.

A. GOGAT activity was eluted with a linear gradient of ethylene glycol (5 to 40%) in the same buffer.

(v) **Step 5: DEAE-Sepharose chromatography.** Fractions that exhibited GOGAT activity were pooled and applied to a DEAE-Sepharose column (23 by 1.7 cm) previously equilibrated with buffer A. Sample elution was performed as described for step 3. Fractions with GOGAT activity were pooled and dialyzed against buffer B.

(vi) **Step 6: affinity chromatography.** A blue Sepharose column (13 by 1.2 cm) was equilibrated with 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, 0.2 mM 2-oxoglutarate, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 50 μg of TLCK per ml (buffer C). After application of the sample obtained from step 5, the column was washed with 20 column volumes of buffer C; GOGAT activity was eluted with buffer C that contained 0.1 mM NADH. Fractions with GOGAT activity were dialyzed against buffer B, concentrated by ultrafiltration with an Amicon YM 30 membrane, and stored at −70°C until used.

Purification of GOGAT from *E. coli*. *E. coli* MX614 (*pro-lac galE ilv-680 thi1*) (3) was grown in minimal medium at 37°C and 250 rpm. Cells were harvested in late exponential phase and stored at −70°C until used. NADPH-GOGAT was purified with modifications to the method of Sakamoto et al. (33). All steps were carried out at 4°C. *E. coli* cells were resuspended in 10 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, and 50 μg of TLCK per ml and disrupted in a Branson sonifier. After streptomycin-sulfate precipitation, the supernatant fluid was dialyzed against 10 mM potassium phosphate (pH 7.2) which contained EDTA and 1 mM DTT. Proteins obtained after 32.5 to 53% ammonium sulfate precipitation were dissolved in 20 mM potassium phosphate (pH 7.2), 1 mM EDTA, 2 mM 2-oxoglutarate, 100 mM KCl, and 1 mM DTT (buffer D) and dialyzed against buffer D without 2-oxoglutarate and DTT (buffer E). The dialyzed fraction was applied to a DEAE Bio-Gel A column equilibrated with buffer D. The column was washed with equilibrating buffer and subsequently eluted with a linear KCl gradient (0.1 to 0.7 M) in the same buffer. Pooled fractions with GOGAT activity were dialyzed against buffer E and applied to a DEAE-Sepharose column previously equilibrated with buffer D. Proteins were eluted and dialyzed as in the preceding step. Then the sample was applied to a red agarose column equilibrated with buffer D. The column was washed exhaustively with equilibrating buffer, and GOGAT activity was eluted with buffer D which contained 0.1 mM NADPH. Pooled fractions were dialyzed against buffer D without KCl and DTT, concentrated with an Amicon YM 30 membrane, and stored at −70°C.

Determination of GOGAT activity. Yeast GOGAT activity was determined by the method described by Roon et al. (30) (5 mM 2-oxoglutarate, 5 mM glutamine, 0.16 mM NADH in 0.1 M potassium phosphate [pH 7.0]), with azaserine (5 mM) inhibition as a control (7). All assays were carried out at pH 7.0. *E. coli* GOGAT activity was determined by the method of Meers et al. (21); controls were also included in the presence of azaserine. Specific activities were expressed as micromoles of NADH oxidized per minute per milligram of protein at room temperature. Protein was measured by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Molecular mass determination. Native molecular mass was determined on a Sephacryl S-300 gel filtration column (2.6 by 90 cm) equilibrated with 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, and 1 mM DTT (buffer F) and eluted at a rate of 6 ml/h. The column was calibrated with molecular mass standards (in kilodaltons) from Sigma Chemical Co. and purified *E. coli* GOGAT (*E. coli* GOGAT, 874 [22]; thyroglobulin, 669; apoferritin, 443; β-amylase, 200; alcohol dehydrogenase, 150; bovine albumin, 66; carbonic anhydrase, 29) dissolved in buffer F which contained 5% glycerol. Molecular mass was determined from a plot of the log molecular mass against volume of distribution/voided volume.

The apparent molecular masses of subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34) with molecular mass standards (in kilodaltons) from Sigma Chemical Co. (myosin, 205; β-galactosidase, 116; phosphorylase *b*, 97.4; bovine albumin, 66; egg albumin, 45; carbonic anhydrase, 29).

Enzyme kinetics. The kinetics of *S. cerevisiae* GOGAT were determined with the enzyme obtained after affinity chromatography (step 6). The enzyme dissolved in buffer B had been frozen at −70°C. K_ms were determined by assaying for GOGAT activity at different concentrations of glutamine, 2-oxoglutarate, or NADH and at saturating concentrations of other substrates (5 mM 2-oxoglutarate, 5 mM glutamine, and 0.16 mM NADH in 0.1 M potassium phosphate [pH 7.0]). Glutamine was used from 0 to 5 mM, and 2-oxoglutarate was used from 0 to 0.5 mM. K_ms were obtained by nonlinear regression analysis of the Michaelis-Menten plot. The K_m for NADH was determined by time course reactions (5), with initial NADH concentrations of 25, 50, and 100 μM. The pH optimum for GOGAT activity was determined with the activity assay buffer adjusted to different pH values with KOH.

Antibody production and Western blot (immunoblot) analysis. Antibodies against the *S. cerevisiae* GOGAT subunit were raised in rabbits and partially purified by ammonium sulfate precipitation according to the method of González-Halphen et al. (12). Immunoblotting was carried out as described by Towbin et al. (41), with the modifications of González-Halphen et al. (11). *S. cerevisiae* GOGAT antiserum was diluted 1:50,000, and goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate was used as the second antibody. *E. coli* large subunit and small subunit GOGAT antisera were diluted 1:3,000,000 and 1:1,000,000, respectively.

TABLE 1. Purification of *S. cerevisiae* NADH-GOGAT[a]

Purification step	Total protein (mg)	Total activity (μmol/min)	Sp act (μmol/min · mg)	Yield (%)	Purification factor[b]
Crude extract	2,900	110	0.04	100	1
30 to 53% $(NH_4)_2SO_4$	860	64	0.07	58	2
DEAE Bio-Gel A	70	22	0.3	20	8
Phenyl-Sepharose	9	9	1.0	8	25
DEAE-Sepharose	1	4	4.0	4	100
Blue Sepharose	0.07	1	14.3	1	358

[a] Based on 49 g (wet weight) cells. Data are representative of four separate purifications.

[b] Fold increase in specific activity.

Amino-terminal sequence analysis. Pure protein was electrophoresed on gels as described by Schägger et al. (34) and transferred onto a ProBlott membrane at 250 mA for 18 h (4°C) in the presence of 10 mM CAPS (pH 11.0)–10% methanol by the procedure of Matsudaira (20). Membranes were stained, destained, and air dried as described by the same author. Amino-terminal sequence analysis was carried out on a Model 470 microsequencer by on-line PTH analysis (Applied Biosystems) at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University.

The search for similarities with amino acid and nucleotide sequences was performed at the National Center for Biotechnology Information by using the BLAST network service.

Specific probes from alfalfa cDNA and heterologous hybridization. In order to obtain specific probes of the amino- and carboxy-terminal regions of alfalfa GOGAT cDNA, two pairs of deoxyoligonucleotides were used for PCR amplification. 5′-GAGTCTCTTGGTCACAAGG-3′, based on the sequence AESL GHK, and 5′-TTTAGTGAAAGGCGGTGCC-3′, based on the sequence KLSL RHC, were used to amplify the amino-terminal region (1,425 bp), and 5′-GC CCTGCACCTTGTGAAG-3′, based on the sequence GRVCPA, and 5′-ATAT GCGAGGCCACTGAGG-3′, based on the sequence EAEQHG, were used to amplify the carboxy-terminal region (765 bp).

Heterologous hybridization between alfalfa GOGAT cDNA probes (probes a and b in Fig. 4A) and *S. cerevisiae* S288C genomic DNA was carried out at 58°C and by washing with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at the same temperature. Probes were radiolabelled by nick translation to a concentration of 10^6 cpm/ml.

PCR amplification of *S. cerevisiae* genomic DNA. The following pair of degenerate deoxyoligonucleotides was designed: 5′-GGGAATTCGC(AGCT)GA (AG)AC(AGCT)CA(CT)C-3′, based on the sequence AETHQT and localized on the putative flavin mononucleotide binding region of alfalfa NADH-GOGAT, and 5′-GGGAATTCAA(AG)TT(AGT)AT(AGCT)AC(AG)T-3′, based on the sequence FNIVHE and 300 bp downstream of the former. Both deoxyoligonucleotides had terminal *Eco*RI restriction sites and two additional guanine residues. PCR amplification of yeast genomic DNA was carried out in a thermocycler (Mj Research, Inc.) with the following program: denaturation at 94°C for 1 min, annealing at 40°C for 3 min, and elongation at 72°C for 1 min. The amplification product (300 bp as judged on an agarose gel) was cloned in the *Eco*RI restriction site of the Stratagene SK plasmid and sequenced by the Sequenase II method.

Independent clones (5×10^4) of the YCp50 *S. cerevisiae* genomic library (31) were screened with the labelled 300-bp PCR insert (10^6 cpm/ml) as the probe.

Northern (RNA) analysis. Northern analysis was carried out as previously described by González et al. (10). Total yeast RNA was prepared from 50 ml of minimal medium overnight cultures of wild-type S288C as described by Struhl and Davis (37).

RESULTS

Purification, kinetics, and immunochemical characterization of GOGAT enzyme. GOGAT enzyme was purified from *S. cerevisiae* CN36 (*MAT*a *his lys1 gdh1*) (7) by classical chromatographic methods (described in Materials and Methods). A 358-fold increase in specific activity was attained (Table 1). SDS-PAGE analysis of the fractions obtained at each step in the purification protocol is shown in Fig. 1A. The molecular mass of the purified subunit was 199 kDa as estimated by SDS-PAGE. In contrast, the *E. coli* GOGAT enzyme was composed of a large subunit (150 kDa) and a small subunit (50 kDa) as described by Miller and Stadtman (24) (Fig. 1B).

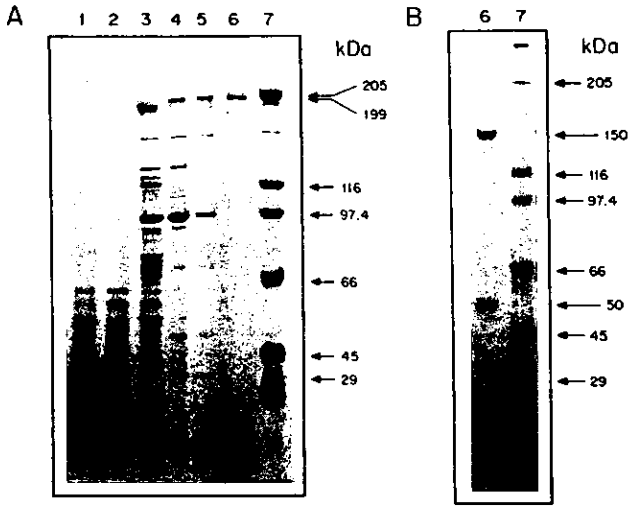

FIG. 1. (A) SDS-6% PAGE of the main fractions obtained during purification of *S. cerevisiae* GOGAT. Lane 1, crude extract (30 μg); lane 2, precipitate obtained with ammonium sulfate (30 to 50%) (30 μg); lane 3, fraction of DEAE Bio-Gel A (30 μg); lane 4, fraction of phenyl-Sepharose column (15 μg); lane 5, fraction from DEAE-Sepharose (4 μg); lane 6, fraction of blue Sepharose (2.5 μg); lane 7, molecular mass standards (myosin [205 kDa], β-galactosidase [116 kDa], phosphorylase *b* [97.4 kDa], bovine serum albumin [66 kDa], ovalbumin [45 kDa], and carbonic anhydrase [29 kDa]). Proteins were visualized with Coomassie blue staining. (B) SDS-6% PAGE of purified *E. coli* NADPH-GOGAT. Lane 6, protein obtained from red agarose (5 μg); lane 7, molecular mass standards (same as in panel A).

The native molecular mass of *S. cerevisiae* GOGAT estimated by gel filtration was 610 kDa (Fig. 2). This value suggests that the protein associates as a trimer. We used the *E. coli* enzyme as a molecular mass standard since it has been shown that *E. coli* GOGAT is arranged as a two-subunit tetramer (24), with a molecular mass of 874 kDa calculated from gene sequence data (27).

Curves for the activity of the purified enzyme plotted against substrate concentration were hyperbolic, showing normal Michaelis-Menten kinetics. The apparent K_ms were 294 μM for glutamine, 104 μM for 2-oxoglutarate, and 3.8 μM for NADH. Activities at saturating substrate concentrations exhibited a broad pH optimum, between pH 6.4 and 7.4 (data not shown).

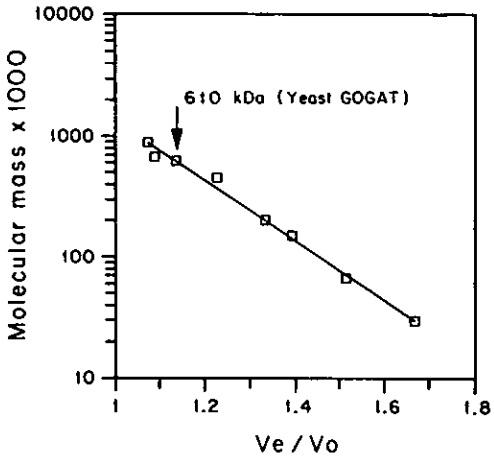

FIG. 2. A native molecular mass of 610 kDa for *S. cerevisiae* GOGAT was determined by regression analysis ($r^2 = 0.989$) of the elution profiles of standard proteins from a Sephacryl S-300 gel filtration column (purified *E. coli* NADPH-GOGAT [874 kDa], thyroglobulin [669 kDa], apoferritin [443 kDa], β-amylase [200 kDa], alcohol dehydrogenase [150 kDa], bovine albumin [66 kDa], and carbonic anhydrase [29 kDa]). V_e/V_0, volume of distribution/voided volume.

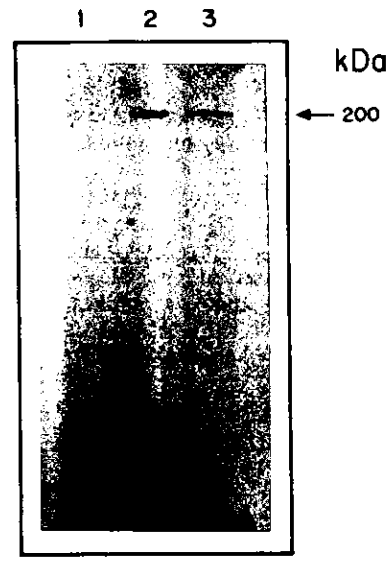

FIG. 3. Western blot with antibodies raised against GOGAT from *S. cerevisiae*. GOGAT anti-serum was probed against 1 μg of purified *E. coli* GOGAT (lane 1) and purified *S. cerevisiae* GOGAT (lane 2) and 50 μg of commercial baker's yeast crude extract with GOGAT activity (lane 3).

The 199-kDa band recovered from the affinity column was transferred to a ProBlott membrane, and the amino-terminal sequence was XGVGFVAN. The initial cysteine was not confirmed by Edman degradation, since the protein had not been previously modified to produce a more stable cysteine derivative (35). Nevertheless, the presence of a cysteine at position 1 of the mature protein was confirmed by nucleotide sequencing of the gene (see Fig. 6B).

Polyclonal antibodies were produced against partially denatured *S. cerevisiae* GOGAT. Western blot analysis of the SDS-PAGE crude extract showed a reactive band that corresponded in molecular mass to that of the purified protein (199 kDa) (data not shown). GOGAT antibodies cross-reacted with baker's yeast crude extracts, also exhibiting a band of 199 kDa (Fig. 3). On the other hand, no reaction was observed with purified *E. coli* GOGAT enzyme (Fig. 3); antibodies produced against each subunit of *E. coli* GOGAT did not cross-react with purified yeast GOGAT (data not shown).

These results suggested that *S. cerevisiae* has a single GOG AT enzyme composed of three 199-kDa monomers. In order to ascertain if a single GOGAT is present in *S. cerevisiae*, we decided to clone the structural gene that codes for this protein.

Cloning of the glutamate synthase structural gene. Since comparative analysis of the amino acid sequences of the alfalfa (NADH-GOGAT), maize (Fd-GOGAT), and *E. coli* (NADPH-GOGAT) enzymes had revealed the existence of conserved regions (14), we explored whether alfalfa GOGAT probes could identify homologous regions in yeast genomic DNA. The amino- and carboxy-terminal regions of the alfalfa GOGAT gene were amplified as described in Materials and Methods and used as probes with *S. cerevisiae* genomic DNA (probes a and b [Fig. 4A]). These probes recognized one or two main bands that were clearly distinguishable from the hybridization background, suggesting that the yeast genome contained sequences homologous to the alfalfa GOGAT gene. Two de-

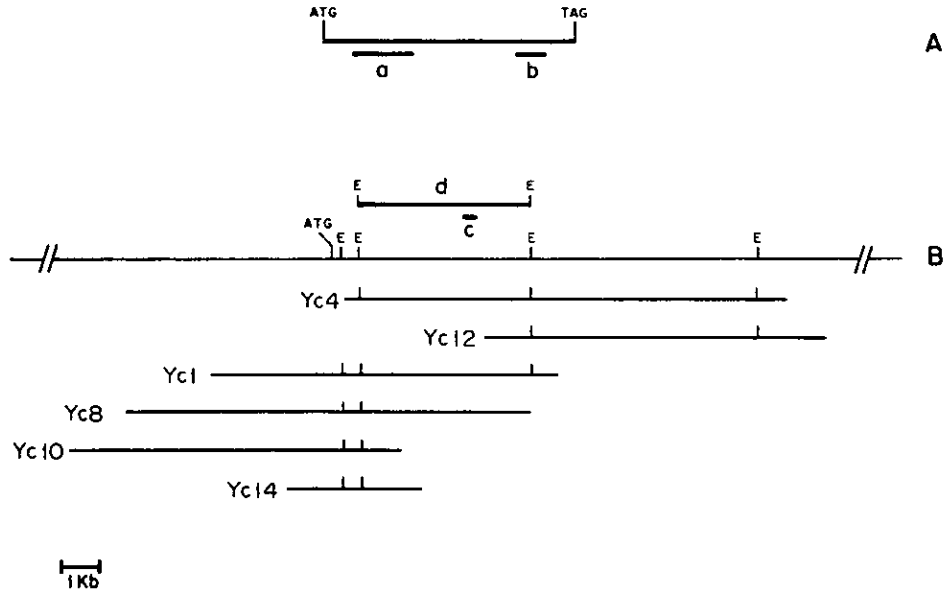

FIG. 4. Restriction map of GOGAT structural gene clones. (A) Diagrammatic representation of cDNA from alfalfa NADH-GOGAT. a, amino-terminal probe; b, carboxy-terminal probe. (B) *Eco*RI restriction map of yeast genomic YCp50 clones that bear the GOGAT structural gene. c, PCR-amplified fragment of *S. cerevisiae* genomic DNA; d, 4.5-kb fragment used for screening the genomic library and for pLV1 construction.

oxyoligonucleotides derived from the central region of the alfalfa GOGAT gene were used to obtain a homologous yeast probe by PCR amplification of *S. cerevisiae* genomic DNA. A 300-bp fragment (probe c [Fig. 4B]) that recognized the restriction fragments previously identified by probes a and b was obtained (Fig. 5). This probe shared 70% similarity at the amino acid level with alfalfa GOGAT, as judged by the Genetic Computer Group program (Fig. 6A).

Probe c was used as the probe for colony hybridization with the YCp50 genomic library. After the first screening, probe d (Fig. 4B) was obtained and used as the probe for colony hybridization with the YCp50 library. After restriction analysis of the plasmids in 20 positive clones, six classes were identified. In order to construct a restriction map (Fig. 4B), probes a and b were hybridized with restriction digests of one plasmid from each class.

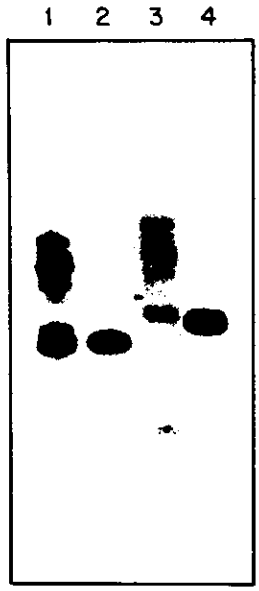

FIG. 5. Southern analysis of *S. cerevisiae* chromosomal DNA. Two micrograms of *S. cerevisiae* chromosomal DNA (wild-type S288C) was digested with *Eco*RI (lanes 1 and 2) or with *Hin*dIII (lanes 3 and 4), loaded on a 0.7% agarose gel, and transferred to a nylon membrane. Lane 1 was hybridized with probe b, lanes 2 and 4 were hybridized with probe c, and lane 3 was hybridized with probe a.

A 0.5-kb fragment defined by a vector and a genomic *Eco*RI restriction site was subcloned from Yc14 (Fig. 4B) and sequenced. The putative amino acid sequence showed that yeast GOGAT protein has a presequence of 28 amino acids and a highly conserved mature amino terminus, compared with the amino-terminal regions of other GOGAT proteins (Fig. 6B). Cys-1 is also conserved; this result is in agreement with the fact that this is an active-site residue involved in the reaction with glutamine (13, 22, 28, 43).

To determine the size of the mRNA that codes for yeast GOGAT, probe c was used on total RNA obtained from a wild-type yeast strain (S288C). As shown in Fig. 7, a major band of around 7 or 8 kb was identified. These results indicate that the GOGAT enzyme from *S. cerevisiae* should be constituted by a high-molecular-weight polypeptide, in agreement with our purification results. In order to analyze whether the 199-kDa polypeptide was the only GOGAT in *S. cerevisiae*, we decided to disrupt the gene (*GLT1*) that codes for this protein.

Construction of a null GOGAT mutant. In order to obtain a null GOGAT mutant, we constructed plasmid pLV1 by cloning the 4.5-kb GOGAT internal fragment (Fig. 4B, probe d) in the YIp5 integrative vector, which harbors the *URA3* yeast gene. After *Bgl*II digestion of pLV1, two fragments were obtained. One was a small (1-kb) internal *Bgl*II-*Bgl*II fragment of the 4.5-kb insert, and the other was the linearized plasmid, bearing at one end a 2-kb GOGAT region and at the other a 1.5-kb GOGAT region. This linearized fragment was used to direct homologous recombination into the yeast genome. In this way, the LA1 (*MATα GDH1 ura3*) haploid strain was transformed to prototrophy. DNAs were isolated from 10 transformants and digested with *Afl*II (an enzyme that does not cut the

A

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S. cerevisiae  VLNDLSVMLL SNVCCKTDGQ LRIGFDIAVA VLLGAESFTL
   M. sativa   -A---R.... GRTTLQ---- -KT-R-V-I- A-----EYGF
     Z. mays   IQ-G-R.... ER-VLRV--G F-S-Q-VLI- AAM--DEYGF
 Antithamnion  -E-S-R.... EKVILRV--G --T-K--II- A-M---E-GF
 Synechocystis ---N-R.... -RIVVE---- MKT-R-V-I- A-----E-GF
   E. coli, LS -A-G-R.... HKIRLQV--G -KT-V--IK- AI------GF
A. brasilense T--R-R.... HR-RLR---G -KT-R--VI- AM----E-GI
   S. oleracea IS-G-R.... ER-ILRV--G -KC-V-VMM- AAM--DEYGF

S. cerevisiae  ATVPLIAMGC VMLRRRCHLN SCAVGIATQD PYLRSKFK
   M. sativa   S-A---TL-- I-M.-K--K- T-P------- -V--E--A
     Z. mays   GS-AM--T-- --A.-I--T- N-P--V-S-R EE--AR-P
 Antithamnion  G--AM--T-- --A.-V--T- N-P--V---R QD--NR-P
 Synechocystis S-A--VSL-- IMM.-A---- T-P------N -E--A--T
   E. coli, LS G-G-MV-L-- KY-.-I---- N--T-V---- DK--KNHY
A. brasilense G-AS------ IMV.-Q--S- T-P--VCV-- DK--Q--V
   S. oleracea GSLAM--T-- --A.-I--T- N-P--V-S-R EE--AR-P
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B

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S. cerevisiae  CGVGFVANKH GEQSHKIVTD ARYLLVNMTH RGAVSSDGN. GDG
   M. sativa   -------ELN -QS-R-T--- -LEM--R--- ---CGCEANT ---
     Z. mays   --------LK NMS-FD--R- -LMA-GC-E- --GCGA-SDS ---
 Antithamnion  -----I--LN SKP-N----E -LNA-SC-E- --GCGA-NIS ---
 Synechocystis -----IVQMK -KV--D--EQ GLQM---LE- ---CGCEP-T ---
   H. vulgare  --------LS N-P-FNV-R- -LTA-GC-E- --GCGA-NDS ---
   E. coli, LS --F-LI-HIE --P---V-RT -IHA-AR-Q- ---ILA--KT ---
A. brasilense -----IAAID -KPRRSV-EK GIEA-KAVW- ----DA--KT ---
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FIG. 6. Amino acid sequence alignment of fragments of *S. cerevisiae* NADH-GOGAT with GOGAT proteins from *Z. mays* (32), an *Antithamnion* sp. (42), *M. sativa* (14), *A. brasilense* (43), *E. coli* (27), *Hordeum vulgare* (2), a *Synechocystis* sp. (NCIB gi/515938), and *Spinacia oleracea* (26). Identical residues are indicated by dashes. Dots indicate gaps introduced to maximize sequence similarity. (A) Yeast PCR-amplified fragment that corresponds to the flavin mononucleotide region of the protein (probe c); (B) amino-terminal region of the mature protein.

construct), and Southern analysis was carried out with the 4.5-kb insert as the probe. The pattern of transformants clearly indicated the insertion of the construct in the wild-type genomic sequence of GOGAT (data not shown). The null mutants obtained were completely devoid of NADH-GOGAT activity. NADPH-dependent GOGAT activity was absent from extracts of both wild-type and GOGAT null mutant strains.

DISCUSSION

Characterization of the yeast gene that codes for GOGAT (*GLT1*) and of its protein product has been reported here.

Our biochemical results show that *S. cerevisiae* has a single oligomeric NADH-GOGAT enzyme composed of three 199-kDa monomers. Our purified preparation has K_ms for glutamine, 2-oxoglutarate, and NADH that are comparable to those reported for plant NADH-GOGAT and for bacterial NADH-GOGAT and NADPH-GOGAT (1, 6, 15, 24, 29, 44). Alignment of the amino-terminal region of our preparation with those of GOGAT enzymes from other species showed high similarity and provided evidence for the unambiguous identification of the purified protein as a GOGAT enzyme. In addition, the yeast GOGAT enzyme consists of a high-molecular-weight monomer similar to the one purified from alfalfa (1). However, although in alfalfa the active enzyme is monomeric, our results suggest that the active enzyme may be homotrimeric in *S. cerevisiae*.

Purification of the yeast enzyme showed that *S. cerevisiae* has a single GOGAT enzyme exclusively composed of 199-kDa polypeptides, thus contradicting two previous observations. As mentioned in the introduction, a heterodimeric GOGAT enzyme composed of a large (169-kDa) subunit and a small (61-kDa) subunit was purified from *S. cerevisiae* (19), and we reported the cloning of a gene (*GUS2*) that was proposed to code for the 61-kDa polypeptide that formed part of the heterodimeric enzyme (10). Thus, the existence of two GOGAT enzymes in *S. cerevisiae*, one homotrimeric and the other heterodimeric, was a possibility to be considered. In order to definitively establish whether this microorganism has a single enzyme or two GOGAT isozymes, we decided to clone the gene that codes for the 199-kDa monomer and to construct null GOGAT mutants.

We have cloned the gene that codes for the high-molecular-weight monomer, since the amino-terminal sequence of the mature protein derived from the nucleotide sequence showed a perfect match with that obtained by direct protein sequencing. Furthermore, the fact that a 7- to 8-kb GOGAT mRNA was identified with the central region of the cloned gene as a probe confirms the existence of a single gene to code for a single 199-kDa polypeptide involved in the GOGAT primary structure.

Disruption of the gene that codes for the 199-kDa polypeptide resulted in a complete lack of NADH-GOGAT activity, thus definitively establishing the fact that *S. cerevisiae* has a single NADH-GOGAT enzyme.

The results presented above strongly suggest that the two-subunit enzyme previously purified from *S. cerevisiae* (19) may have been the result of partial proteolytic degradation, as has

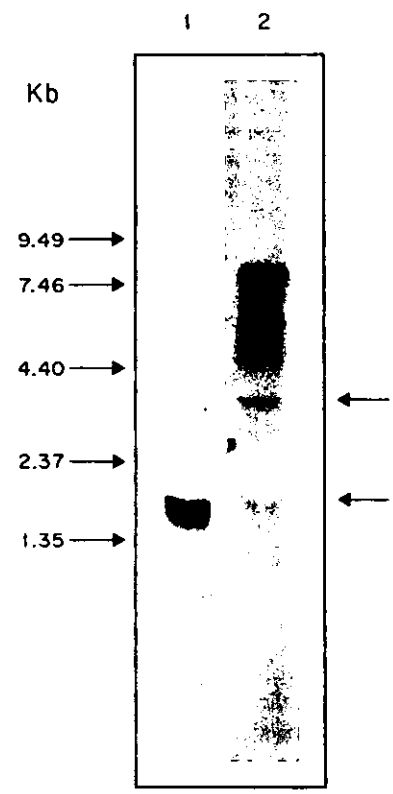

FIG. 7. Northern blot of total RNA obtained from wild-type S288C. Total RNA samples (10 μg each) were separated by electrophoresis on a denaturing 1% agarose gel and transferred to a Hybond N filter (see Materials and Methods). RNA filters were probed with the *Bam*HI-*Hin*dIII actin fragment from plasmid pYA102 (8) (lane 1) or probe c (Fig. 4B) (lane 2). Arrows on right indicate the migration positions of 25S and 16S rRNAs. Arrows on left correspond to a 0.4- to 9.4-kb RNA ladder from GIBCO BRL. Autoradiograms were exposed for 1 and 48 h (lanes 1 and 2, respectively). The specific activity of each probe was about 10^9 cpm μg^{-1} DNA.

been reported for maize leaf Fd-GOGAT (32) and for Fd-GOGAT from a *Synechococcus* sp. (18). Since the dimeric enzyme was purified from baker's yeast, we decided to explore whether the 199-kDa polypeptide was present in crude extract from this source. Our results unambiguously show that there is a 200-kDa band in baker's yeast extracts that cross-reacts with the antibodies raised against our purified enzyme.

We previously reported the cloning of the *GUS2* gene and proposed that this gene coded for the 61-kDa GOGAT subunit (10). This proposition was based mainly on the fact that *E. coli* GOGAT mutants which lacked the small subunit of GOGAT were complemented by *GUS2*. However, this result proved to be irreproducible. Furthermore, since we have shown that the only polypeptide involved in GOGAT structure is encoded by a 7- to 8-kb mRNA, the possibility that *GUS2*, which codes for a 1.5-kb mRNA, plays a role in GOGAT structure is completely ruled out. However, the fact that *GUS2* complemented our previously isolated GOGAT-less mutant (CN39) (10) suggests that the product of *GUS2* plays a regulatory role and that the mutations present in strain CN39 are regulatory. Experiments are under way in order to address this matter.

The finding that *S. cerevisiae* has a eukaryotic-type NADH-GOGAT is in agreement with the fact that all of the eukaryotic NADH-GOGAT enzymes purified so far are composed of high-molecular-weight polypeptides. This should shed light on studies of the evolutionary origins of different GOGAT genes.

Finally, it is worth mentioning that, like GOGAT proteins from other organisms (14, 32), the yeast GOGAT enzyme contains a presequence, although this region is not conserved; its role remains to be studied.

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Capítulo III

Sequence of the *GLT1* gene from *Saccharomyces cerevisiae* reveals the domain structure of yeast glutamate synthase.

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Yeast (1996) **12:**1359-1366.

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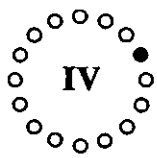

Yeast Sequencing Reports

Sequence of the *GLT1* Gene from *Saccharomyces cerevisiae* Reveals the Domain Structure of Yeast Glutamate Synthase

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Glutamate synthase (GOGAT) and glutamine synthetase play a crucial role in ammonium assimilation and glutamate biosynthesis in the yeast *Saccharomyces cerevisiae*. The GOGAT enzyme has been purified and the GOGAT structural gene (*GLT1*) has been cloned, showing that this enzyme is a homotrimeric protein with a monomeric size of 199 kDa.

We report the *GLT1* nucleotide sequence and the amino acid sequence of its deduced protein product. Our results show that there is a high conservation with the corresponding genes of *Escherichia coli*, *Medicago sativa* (alfalfa) and *Zea mais* (maize). Binding domains for glutamine, cofactors (FMN and NADH) and the cysteine clusters (which comprise the iron-sulfur centres) were tentatively identified on the basis of sequence comparison with GOGAT sequences from *E. coli*, alfalfa and maize. The sequence of *GLT1* has been deposited in the EMBL data library under Accession Number X89221.

KEY WORDS — glutamate synthase; *S. cerevisiae*; chromosome IV

INTRODUCTION

Two pathways for glutamate biosynthesis have been found in several microorganisms (Bastarrachea *et al.*, 1980; Hummelt and Mora, 1980; Marques *et al.*, 1992; Senior, 1975; Tempest *et al.*, 1970) and in higher plants (Anderson *et al.*, 1989; Chen and Cullimore, 1988; Hayakawa *et al.*, 1992; Miflin *et al.*, 1980; Sakakibara *et al.*, 1991; Suzuki *et al.*, 1982). One pathway requires only the action of the NADP-dependent glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4), while the other one involves the concerted action of glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.13).

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GOGAT is an iron-sulfur flavoprotein (Miller and Stadtman, 1972) which catalyses the reductive transfer of the amide group of L-glutamine to 2-oxoglutarate, resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The reducing equivalents are provided by NADH, NADPH or by reduced ferredoxin (Fd). Bacterial GOGATs have been extensively studied and, in all cases, it has been shown that their oligomeric structure is composed of two different polypeptides (Miller and Stadtman, 1972; Trotta *et al.*, 1974; Adachi and Suzuki, 1977; Schreier and Bernlohr, 1984; Ratti *et al.*, 1985; Wang and Nicholas, 1985; Carlberg and Nordlund, 1991). One case has been reported that describes a bacterial GOGAT built up of five different

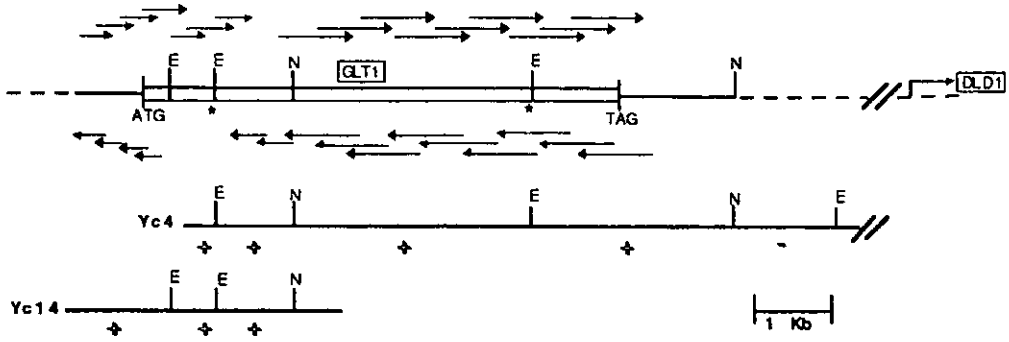

Figure 1. Sequencing strategy of the *S. cerevisiae GLT1* gene. Only the relevant sites are indicated. E, *Eco*RI; N, *Nsi*I. The arrows indicate the polarity and extent of sequencing. Two asterisks mark the *Eco*RI-*Eco*RI 4·5 kb fragment previously used for gene disruption. The plus signs (+) indicate the restriction fragments that recognize *GLT1* mRNA.

subunits (Singhal *et al.*, 1989). Most of the bacterial enzymes use NADPH as a cofactor, although two NADH-dependent GOGAT's have been found (Wang and Nicholas, 1985; Singhal *et al.*, 1989). In some cases, the genes that code for the large and small polypeptides have been cloned and sequenced (Oliver *et al.*, 1987; Vanoni *et al.*, 1990; Pelanda *et al.*, 1993).

In higher plants, GOGAT occurs in two forms that differ in molecular mass, kinetics, locations within the plant and cofactor specificity (Suzuki and Gadal, 1984). The genes coding for NADH-GOGAT from alfalfa (*Medicago sativa*; Gregerson *et al.*, 1993) and Fd-GOGAT from various plants have been cloned (Sakakibara *et al.*, 1991; Avila *et al.*, 1993; Nalbantoglu *et al.*, 1994). Comparative analysis of the amino acid sequences of the GOGAT structural genes from *M. sativa* and *Zea mays* with the genes coding for the subunits that build up the heterodimeric GOGAT of *Escherichia coli* revealed highly conserved regions (Gregerson *et al.*, 1993). The presequences of these proteins show reduced similarity. Although conserved regions are found throughout the length of the mature protein, three regions involved in cofactor binding have been defined (Knaff *et al.*, 1991; Pelanda *et al.*, 1993), and one region has been found that could be involved in glutamine binding (Pelanda *et al.*, 1993).

A protein bearing GOGAT activity has also been purified from fungi. In *Neurospora crassa*, a monomeric GOGAT consisting of a 200 kDa polypeptide was purified (Hummelt and Mora, 1980), indicating that in contrast to what had been found in prokaryotic organisms, eukaryotic microorganisms had GOGAT enzymes which were built up of high molecular weight subunits similar to those present in higher plants. However, in the yeast *Saccharomyces cerevisiae*, a heterodimeric GOGAT enzyme composed of a large (169 kDa) and a small (61 kDa) subunit of the prokaryotic type was purified (Masters and Meister, 1982). Further analysis of the structure of the GOGAT enzyme from *S. cerevisiae* revealed the existence of an homotrimeric protein built up of plant-like high-molecular-weight polypeptides (Cogoni *et al.*, 1995). In order to analyse whether this yeast had two different GOGAT enzymes, the structural gene(s) were cloned. It was found that this yeast had a single GOGAT gene (*GLT1*) coding for a 199 kDa polypeptide. *GLT1*-disrupted 'null' mutants were completely devoid of GOGAT activity (Cogoni *et al.*, 1995). These results clearly show that in yeast there is a single homotrimeric GOGAT enzyme, thus supporting the proposition that eukaryotic GOGAT enzymes were monomers or oligomers built up by high-molecular-weight polypeptides. The sequences of the *GLT1* gene from *S. cerevisiae* and its deduced protein product (*GLT1p*) are reported here. Our results show that the previously defined functional domains for the binding of cofactors and glutamine by glutamate synthase (Pelanda *et al.*, 1993; Gosset *et al.*, 1989) are highly conserved in *GLT1p*.

MATERIALS AND METHODS

GLT1 subcloning

Plasmids Yc4 and Yc14 were isolated from a yeast genomic library (Cogoni *et al.*, 1995). A 6·5 kb *Nsi*I-*Nsi*I fragment was obtained from Yc4

and subcloned in *Pst*1-digested plasmid SK (pYc4 N1). The obtained plasmid was digested with *Eco*RI and religated on itself (pYc4N2), while the excised fragment containing a genomic and a plasmid-derived *Eco*RI-restriction site was cloned in SK (pYc4N3). The sequencing of the latter two plasmids was performed automatically using *Taq* DNA polymerase and fluorescent-dideoxy terminators in a cycle sequencing method. The resultant DNA fragments were electrophoresed and analysed using an automated Applied Biosystems 373A DNA sequencer (in the W. M. Klerk facility at Yale University). These fragments covered 70% of the *GLT1* sequence. The remaining 30%, which contained the 5′ end of the gene, was sequenced manually from plasmid Yc14 by the di-deoxy sequencing method (Sanger *et al.*, 1977) using the USB sequenase kit, version 2.0. The oligonucleotides used to perform the sequence are depicted in Figure 1.

Amino acid computer analysis

For the search of homologies of the deduced *GLT1p* amino acid sequence, we used the GCG software package (Pearson and Lipman, 1988). In particular, Fasta (on GenBank and SwissProt), Bestfit, Findpattern and Prettyplot were used to analyze the sequences.

RESULTS AND DISCUSSION

The use of heterologous gene amplification to isolate clones harbouring the GOGAT structural gene (*GLT1*) from a yeast genomic library has been described previously (Cogoni *et al.*, 1995). Two experimental approaches were used in order to establish definitively that these clones carried the GOGAT structural gene. On the one hand, a 4·5 kb fragment obtained from the Yc4 clone was used to obtain null mutants in the corresponding homologous gene. Southern analysis of the obtained mutants showed that insertion had occurred in the GOGAT structural gene, and the mutants completely lacked GOGAT activity. On the other hand, the amino-terminal sequence of the GOGAT enzyme was compared to that derived from the nucleotide sequence of *GLT1*. An identical region was found 53 amino acids downstream of the first in-frame methionine. These data indicated that, like all the other GOGATs so far studied, the yeast enzyme has a presequence (Cogoni *et al.*, 1995). The restriction fragments,

```
   1 MFVLKSDNFD PLEEAYEGGT IQHYNDEHHL HKSWANVIFD KRGLYDFDYE
  51 HDACGVGFVA NKHGECSHKI VTDARYLLVN MTHHGAVSSD GNGDGAGILL
 101 GIPHEFMKRE FKLDLDLDIP EMGKYAVGNV FPKKNEKNNK KNLIKCQKIF
 151 EDLAASFNLS VIGWRTSRRF YYLGDVALSR EPTILQPLLV PLYDEKQEEF
 201 NETKFRTQLY LLRKEASLQI GLENWFYVCS LNNTTIVYKG QLTPAQVYNY
 251 YPDLTNAHFK SHMALVHSRF STNTFPSWDR AQPLRWLAHN GEINTLRGNK
 301 NWMRSREGVM HSATFKDELD KLYPIIEEGG SDSAALDNVL ELLTINGTLS
 351 LPEAVMMVP EAYHKDMDSD LKAWYDMAAC LMEPWDGPAL LTFTDGRYCG
 401 AILDRNGLRP CRYYITSDDR VICASEVGVI PIENSLVVQK GKLKPGDLIP
 451 SDTQLGEMVD TKKLKSQISK RQDFKSWLSK VTKLDDLLSK TANLVPKEFI
 501 SQDSLSLKVQ SDPRLLANGY TFEQVTFLLT PMALTGKEAL GSMGNDAPLA
 551 CLNENPVLLY DYFRQLFAQV TNPPIDPIRE ANVMSLECTV GPQGNLLEMH
 601 SSQCDRLLLK SPILHWNEPQ ALKNIEAAYP SWSVAEIDIT FDKSEGLLGY
 651 TDTIDKITKL ASEAIDDGKK ILIITDRKMG ANRVSISSLI AISCIHHHLI
 701 RNKQRSQVAL ILETGEAREI HHFCVLLGYG CDGVYPYLAM ETLVRMNREG
 751 LLRNVNNDND TLEEGQILEN YKKAIDAGIL KVMSKMGIST LASYKGAQIF
 801 EALGLDNSIV DLCFTGTSSR IRGVTFEYLA QDAFSLHERG YPSRQTISKS
 851 VNLPESGEYH FRDGGYKHVN EPTAIASLQD TVRNKNDVSW QLYVKKEMEA
 901 IRDCTLRGLL ELDFENSVSI PLEQVEPWTE IARRFASGAM SYGSISMEAH
 951 STLAIAMNRL GAKSNCGEGG EDAERSAVQE NGDTMRSAIK QVASARFGVT
1001 SYYLSDADEI QIKIAQGAKP GEGGELPAHK VSKDIAKTRH STPNVGLISP
1051 PPHHDIYSIE DLKQLIYDLK CANPRAGISV KLVSEVGVGI VASGVAKAKA
1101 DHILVSGHDG GTGAARWTSV KYAGLPWELG LAETHQTLVL NDLRRNVVVQ
1151 TDGQLRTGFD IAVAVLLGAE SFTLATVPLI AMGCVMLRRC HLNSCAVGIA
1201 TQDPYLRSKF KGQPEHVINF FYYLIQDLRQ IMAKLGFRTI DEMVGHSEKL
1251 KKRDDVNAKA INIDLSPILT PAHVIRPGVP TKFTKKQDHK LHTRLDNKLI
1301 DEAEVTLDRG LPVNIDASII NTDRALGSTL SYRVSKKFGE DGLPKDTVVV
1351 NIEGSAGQSF GAFLASGITF ILNGDANDYV GKGLSGGIIV IKPPKDSKFK
1401 SDENVIVGNT CFYGATSGTA FISGSAGERF GVRNSGATIV VERIKGNHAF
1451 EYMTGGRAIV LSQMESLNAF SGATGGIAYC LTSDYDDFVG KINKDTVELE
1501 SLCDPVELAF VKNLIQEHWN YTQSDLAARI LGNFNHYLKD FVKVIPTDYK
1551 KVLLKEKAEA AKAKAKATSE YLKKFRSNQE VDDEVNTLLI ANQKAKEQEK
1601 KKSITISNKA TLKEFKVVDL EDAVPDSKQL EKNSERIEKT RGFMIHKRRH
1651 ETHRDPRTRV NDWKEFTNPI TKKDAKYQTA RCMDCGTPFC LSDTGCPLSN
1701 IIPKFNELLF KNQWKLALDK LLETNNFPEF TGRVCPAPCE GACTLGIIED
1751 PLGIKSVERI IIDNAFKEGW IKPCPPSTRT GFTVGVIGSG PAGLACADML
1801 NRAGHTVTVY ERSDRCGGLL MYGIPNMKLD KAIVQRRIDL LSAEGIDFVT
1851 NTRIGKTISM DELKNKHNAV VYAIGSTIPR DLPIKGRELK NIDFAMQLLE
1901 SNTKALNKD LEIIPEKIQG KKVIVVGGGD TGNDCLGTSV RHGAASVLNF
1951 ELLPEPPVER AKDNPWPQWP RVMRVDYGHA EVKPHYGRDP REYCILSKEF
2001 IGNDEGEVTA IRTVRVEWKK SQSGVWQMVE IPNSEEIFEA DIILLSMGFV
2051 GPELINGNDN EVKKTRRGTI ATLDDSSYSI DGGKTFACGD CRRGQSLIVW
2101 AIQEGRKCAA SVDKFLMDGT TYLPSNGGIV QRDYKLLKEL A
```

Figure 2. Deduced amino acid sequence of *GLT1p*. The asterisks indicate conserved residues involved in glutamine binding of amidotransferases, the region of FMN binding is underlined, the small circles indicate cysteine clusters, the # signs indicate the conserved residues involved in NADH binding. The cysteine 54 is the first residue of the mature GOGAT.

obtained from Yc4 and Yc14, which were able to recognize a 7 kb *GLT1* mRNA when used as probes on total yeast RNA are indicated in Figure 1. To obtain the full *GLT1* gene sequence,

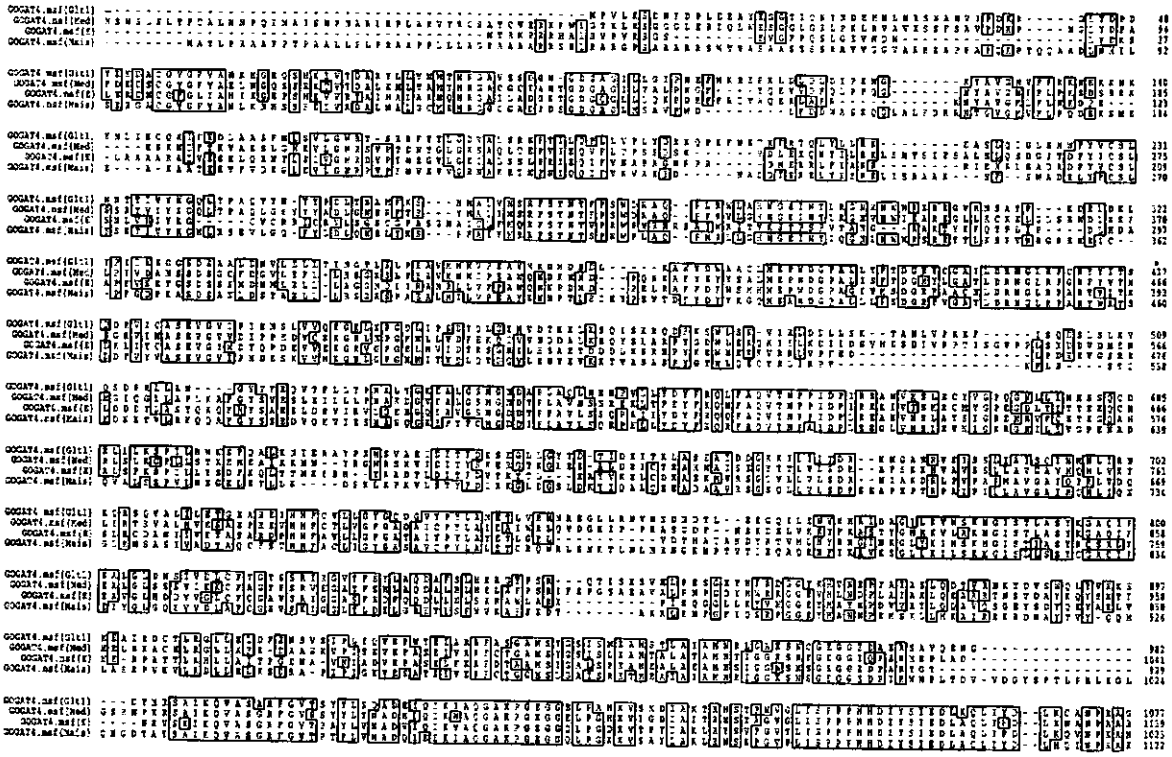

Figure 3. Amino acid sequence alignment of the predicted *S. cerevisiae* GOGAT protein with that of *M. sativa*, *E. coli* and *Z. mais* obtained using the Prettyplot program of the GCG package. The *E. coli* sequence from amino acids 1 to 1514 belongs to the large subunit (*gltB*) and that from 1515 to 1984 represents the small subunit (*gltD*).

these regions were subcloned and sequenced using the oligonucleotides shown in Figure 1.

The 7550 bp of genomic DNA we sequenced (GenBank accession number X89221) revealed a putative TATA box (nt 57), a first in-frame ATG (nt 263) long open reading frame (6423 bp), coding for a putative protein of 2141 amino acids (shown in Figure 2) and a TAG terminator. Like most yeast genes *GLT1* does not contain introns. Computer comparison of further sequence obtained from the longer clone (Yc14) we isolated previously (Cogoni *et al.*, 1995) revealed the presence, 1625 bp away from the TAG stop codon of *GLT1*, of the already sequenced *DLD* gene. Since this gene has been mapped on chromosome IV (Lodi *et al.*, 1992) and is present on the GenBank chromosome IV partial sequence, we can assign *GLT1* to this chromosome.

The alignment shown in Figure 3 was obtained using the PRETTY-PLOT program of GCG. For clarity, we have only included three of the GOGATs so far sequenced. These were chosen in order to compare our sequence with a representative of each one of the GOGAT classes that have been studied (prokaryotic, and eukaryotic NAD(P)H- or Fd-dependent). The degree of amino-acid similarity between the *S. cerevisiae* GOGAT and the other sequenced GOGATs ranged from 58% (*E. coli*) to 69·6% (*M. sativa*), revealing a high degree of conservation among bacteria, fungi and plants.

As mentioned in the Introduction, *E. coli* GOGAT is an heteroctamer consisting of large (153 kDa) and small (51 kDa) subunits, which are encoded by the *glt*B and *glt*D genes, respectively; both belonging to the same operon. Yeast GOGAT shows homology with both *E. coli* subunits (Figures 3 and 4), similar to what has been found for the NADH-dependent protein from *M. sativa*. Conversely, all sequenced Fd-dependent GOGATs lack a region corresponding to the bacterial small subunit. These results indicate that, as has been previously proposed, the NADH binding domain should reside in a region corresponding to the bacterial small GOGAT subunit (Gosset *et al.*, 1989).

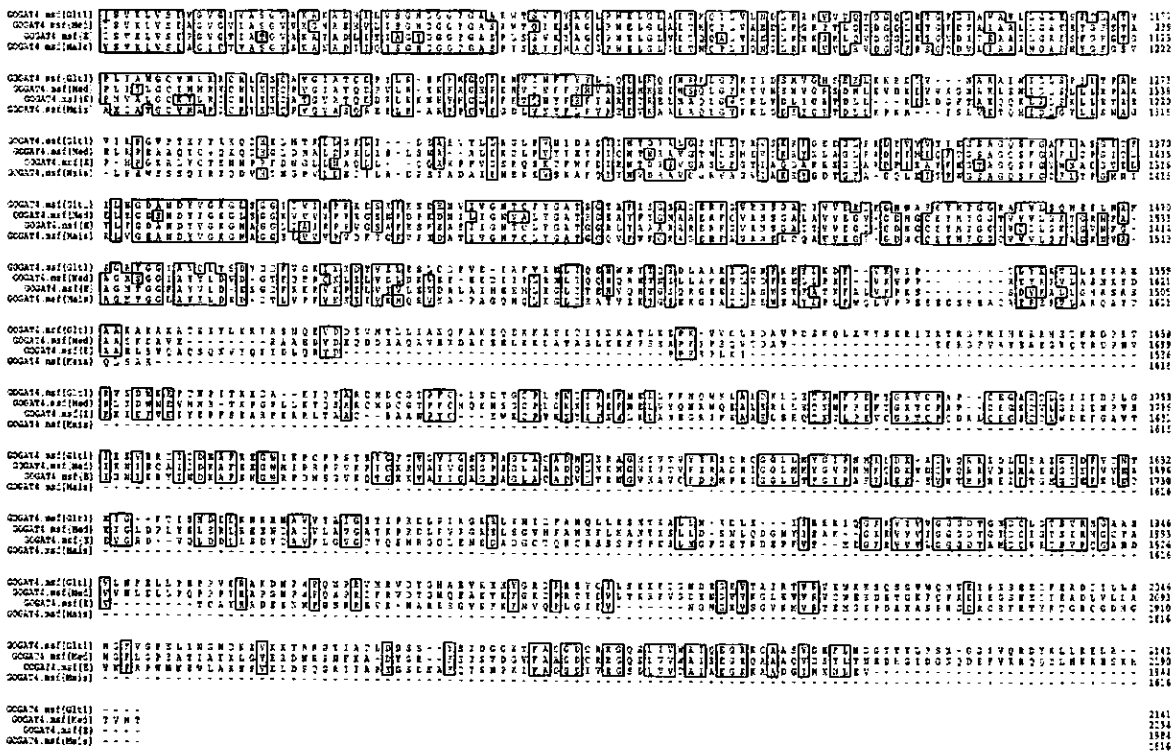

Figure 3. *Continued.*

S. cerevisiae *glutamate synthase relevant functional domains*

Relevant functional domains of the yeast GOGAT polypeptide were deduced by amino acid sequence comparison with previously sequenced genes, using GCG programs. Figure 4 shows a comparison of the organization of the functional domains of *GLT1p* with those of the NADH-dependent *E. coli* and *M. sativa* GOGATs and with that of the Fd-dependent enzyme from *Z. mais*. It is evident that the position of functional domains in the yeast protein reflects the organization of the *E. coli gltBD* operon, in which the gene coding for the large subunit is located immediately after the promoter region. This feature has also been found in other eukaryotic organisms (Gregerson *et al.*, 1993).

Glutamine amidotransferase domains In bacteria, it has been shown that the glutamine amidotransferase domain, which is involved in the transfer of the amide group from L-glutamine to 2-oxoglutarate in order to yield L-glutamate, is located in the large subunit (Pelanda *et al.*, 1994). In yeast, this region was found at the amino terminus of the protein. The amino acid sequence comparison, carried out among glutamine-binding enzymes of several prokaryotic organisms, has suggested two possible consensus motifs for the binding domain (Pelanda *et al.*, 1993). The first one, derived from the sequence alignment of several amidotransferases, which is 13 amino acids long (L/I, P, I, L/I/F, G, T, C, L, G, H, Q, A, I) gave a fairly weak homology with both the *E. coli* GOGAT (amino acids 236–249) and *S. cerevisiae* GOGAT (amino acids 261–270). Instead a strong similarity was found with a second glutamine-binding motif derived from the alignment of various phosphoribosyl pyrophosphate amidotransferase (Pelanda *et al.*, 1994). As in the case of the other GOGATs, a conserved cysteine residue is present as the first amino acid of the mature protein (Figure 2) and it seems to be part of the glutamine-binding domain (Pelanda *et al.*, 1994), thus suggesting that enzyme activation, upon the cleavage of the preprotein, could depend on the exposure of the cysteine number 1 residue.

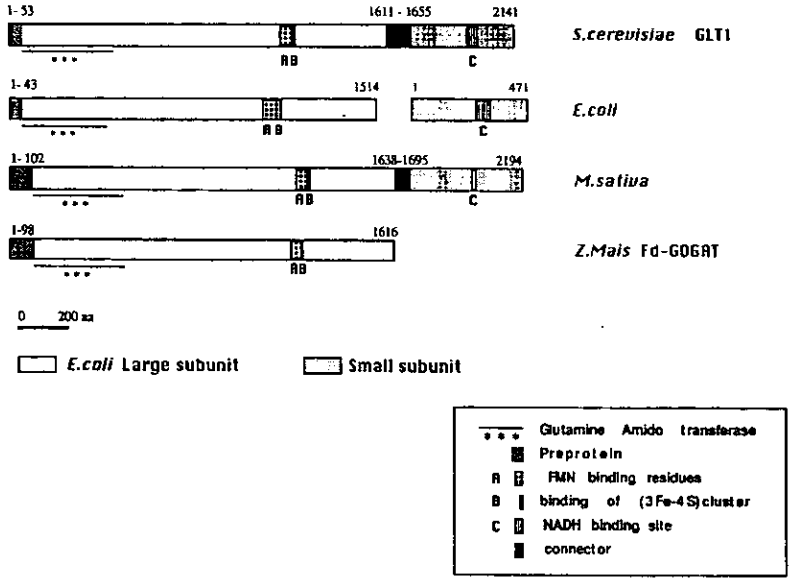

Figure 4. Comparison of *S. cerevisiae* GOGAT functional domains with that of *M. sativa*, *E. coli* and *Z. mais*.

Potential iron-sulfur binding centres and other cofactor binding sites It has been shown that the glutamate synthase is an iron-sulfur flavoprotein and that the bacterial glutamate syntheses have three distinct iron-sulfur centres per $\alpha\beta$ promoter (Vanoni *et al.*, 1992). In *S. cerevisiae* GOGAT, a canonical cysteine cluster for the binding of [3Fe-4S] is present at amino acids 1184, 1190 and 1195 in a highly conserved region. Two other cysteine clusters, also conserved, are present on the carboxy-terminal region of the *GLT1p* (amino acids 1682, 1685, 1690, 1696 and 1735, 1739, 1743); these could also be involved in the electron transfer process from NADH to the site where the reductive amide transfer takes place.

The first consensus sequences for the binding of flavine and pyridine cofactors to the *E. coli* GOGAT were obtained by comparison with known sequences of flavoproteins and NADH-dependent dehydrogenases (Mathews, 1992). It is worth noting that there is a particularly high degree of conservation between a region in the *E. coli* GOGAT (amino acids 1077 to 1134 of the large subunit) and a yeast flavocytochrome b_2 glycine-rich region (amino acids 388 to 444), which has been proposed to be involved in the binding of the phosphate group of the cofactor (Lederer *et al.*, 1985). It has been shown that the flavocytochrome b_2 carries one molecule of FMN. The same region has been identified in plant GOGATs (Sakakibara *et al.*, 1991; Gregerson *et al.*, 1993) and our results show that this region is also present in the *GLT1p* amino acid sequence (Figure 2, amino acids 1131–1188).

The GOGAT enzyme from yeast shows five highly conserved amino acids at positions 1927, 1929, 1932, 1943 and 1951, previously proposed as candidates for the NADH binding sites (Scrutton *et al.*, 1990) and found to be present in *M. sativa* enzyme (Gregerson *et al.*, 1993). As expected, this region has not been found in maize GOGAT protein which, as mentioned earlier, is an Fd-dependent enzyme.

Two other regions have been identified in the *GLT1* amino acid deduced sequence: a so-called 'connector' (amino acids 1567–1658) units and a carboxy-terminal sequence of 23 amino acids not present in the *E. coli* enzyme. *GLT1* shows homology with both regions of the corresponding sequence of *M. sativa* GOGAT.

Glutamate synthase presequence Like all the other glutamate synthase proteins, yeast GOGAT is preceded by a transit peptide 53 amino acids long. A possible role for this presequence is to function as a mitochondrial targeting signal. However, the common characteristics of the mitochondrial import signals, which are: the preponderance of

positively charged residues, the near absence of negatively charged amino acids and a higher than normal content of hydroxy residues, are absent in the *GLT1* presequence. Conversely, the GOGAT presequence has a net charge of −8 and only two serines and four leucines over the 53 residues. As well as the *M. sativa* GOGAT (Gregerson *et al.*, 1993), the yeast protein shows an unusually high content of glutamic and aspartic residues. However, in the case of *M. sativa*, the net charge of +4 and the high content of hydroxy amino acids, plus the fact that in plants proteins can be transported not only to mitochondria but also to chloroplasts and to amyloplasts, leaves an ambiguous interpretation regarding the possible role of the presequence. In the case of *S. cerevisiae* GOGAT it seems more probable that the presequence is required in order to have a reserve of inactive GOGAT in the cell that could be quickly activated by the cleavage of the presequence and the consequent exposure of the amino-terminal cysteine of the mature enzyme required for the glutamine binding.

Our results show that the glutamate synthase amino acid sequence and the spatial localization of the different functional domains are highly conserved from prokaryotes to eukaryotes. Considering the organization of the GOGAT prokaryotic operons, it could be speculated that in the transition from bacteria to yeast, the enzyme became a single polypeptide. This probably occurred by gene fusion of the two structural genes present in the same order in all the prokaryotic operons containing the GOGAT coding genes so far studied, with the only exception of the *Azospirillum brasiliense* operon, which presents an inversion of the two genes.

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Capítulo IV

Regulation of expression of *GLT1*: the gen encoding glutamate synthase in *Saccharomyces cerevisiae.*

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Title: Regulation of Expression of *GLT1*, the Gene Encoding Glutamate Synthase in *Saccharomyces cerevisiae*

The above manuscript has been accepted for publication in the *Journal of Bacteriology* by Dr. Dan Fraenkel, Microbiology and Molecular Genetics, Building D1, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115-5701 (phone 617-432-4437, fax 617-738-7664). It has been scheduled to appear in the Eukaryotic Cells section of the July '98 issue (volume 180, issue 14) with the implicit understanding that the signed copyright transfer agreement be returned within 3 days of receipt and that the authors will remit the page charges of $50 per page for the first six pages and $75 for each page in excess of six.

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TITLE:

REGULATION OF EXPRESSION OF *GLT1*, THE GENE ENCODING GLUTAMATE SYNTHASE IN *Saccharomyces cerevisiae*.

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ABSTRACT

Saccharomyces cerevisiae glutamate synthase (GOGAT) is an oligomeric enzyme composed of three 199-kDa identical subunits encoded by *GLT1*. The present work analyses *GLT1* transcriptional regulation. *GLT1-lacZ* fusions were prepared and *GLT1* expression was determined in a *GDH1* wild type strain and in a *gdh1* mutant derivative grown in the presence of various nitrogen sources. Null mutants impaired in *GCN4*, *GLN3*, *GAT1/NIL1* or *UGA43/ DAL80* were transformed with a *GLT1-lacZ* fusion in order to determine whether the above mentioned transcriptional factors had a role in *GLT1* expression. A collection of increasingly larger 5' deletion derivatives of the *GLT1* promoter was constructed in order to identify DNA sequences that could be involved in *GLT1* transcriptional regulation. The effect of the lack of *GCN4*, *GLN3*, or *GAT1/NIL1* was also tested in the pertinent 5' deletion derivatives. Our results indicate that: i) *GLT1* expression is negatively modulated by glutamate mediated repression and positively regulated by Gln3p and Gcn4p-dependent transcriptional activation; ii) Two cis acting elements, a CCG-N_{15}-CGG palindrome and an imperfect poly (dA-dT) were identified, which could play a role in *GLT1* transcriptional activation; iii) *GLT1* expression is moderatly regulated by *GCN4* under amino acid deprivation. Our results suggest that in a wild type strain grown on ammonium, GOGAT constitutes an ancillary pathway for glutamate biosynthesis.

INTRODUCTION

The existence of two pathways for glutamate biosynthesis has been demonstrated in a variety of organisms. In one pathway, $NADP^+$-dependent glutamate dehydrogenase ($NADP^+$-GDH; EC 1. 4. 1. 4) catalyses the reductive amination of 2-oxoglutarate to form glutamate (24). The existence of an alternative pathway for the net biosynthesis of glutamate, was demonstrated by Tempest et al.(45). In this pathway, glutamate is aminated to form glutamine by glutamine synthetase (GS; EC 1. 4. 1. 13), the amide group of which is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT; EC1.4.1.13), resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The GS-GOGAT pathway has been found in several microorganisms (8, 25, 30, 32, 40) and in higher plants (32). In *S. cerevisiae*, besides the $NADP^+$-GDH1 encoded by *GDH1* and GOGAT encoded by *GLT1* (18, 24, 33), there is a third route for glutamate biosynthesis, constituted by a $NADP^+$-GDH1 isozyme ($NADP^+$-GDH3), encoded by *GDH3* (2). Thus in this microorganism, mutations inactivating *GDH1*, *GLT1* and *GDH3* are needed in order to attain full glutamate auxotrophy (2).

The presence of multiple pathways for glutamate biosynthesis in several microorganisms, has stimulated discussion on the need for several routes for the biosynthesis of the same end product. Since the demonstration of the existence of GOGAT as an alternative pathway for glutamate biosynthesis (45), it was proposed, that the role of the GS-GOGAT pathway

would be that of ammonium assimilation and glutamate biosynthesis under ammonium limitation (45). In fact, it has been shown that for *Klebsiella aerogenes* this was the case (40). However, in other microorganisms, (18, 28, 40) $NADP^+$-GDH is used to incorporate ammonia during either nitrogen limitation or nitrogen excess. Thus, the initial hypothesis suggesting the differential utilization of $NADP^+$-GDH vs GS-GOGAT pathways under excess or limiting ammonia, does not hold for most of the microorganisms so far studied. Since in most cases $NADP^+$-GDH seems to be the main pathway for glutamate biosynthesis, the role of GOGAT remains unclear. Physiological studies have been performed in either wild type or mutant strains impaired in GOGAT or in $NADP^+$-GDH activity. In some cases, this approach has allowed the proposal of different roles for GOGAT in different microorganisms (3, 20, 23, 25, 28, 46). Few studies have been done in order to understand the regulation of GOGAT encoding genes, which could also provide information regarding to the possible role of this enzyme in glutamate biosynthesis. In the case of *E. coli*, it has been found that the two structural genes (*gltB* and *gltD*) coding for the two *E. coli* GOGAT subunits form an operon with a third regulatory gene *gltF* (9), being involved in the glutamate mediated repression of the *gltBDF* operon (10). In addition, the *E. coli gltBDF* operon appears to be transcriptionaly regulated by the leucine-responsive regulatory protein (Lrp) (16). In *Bacillus subtilis*, GOGAT gene expression (*gltA*, *gltB*) is dependent on a positive regulator (*gltC*) that is itself transcribed from a divergent but overlapping promoter site. (6). It has been postulated that

the product of *gltC* is a positive transcription factor that acts at the *gltA* promoter to stimulate transcription under conditions of limiting glutamate (7).

During the last years, our group has been interested in defining and understanding the role of each one of the pathways involved in glutamate biosynthesis in *S. cerevisiae* (2, 17, 18). Since as mentioned earlier, in this yeast there are three pathways for glutamate biosynthesis and the precise function of each one has not been established, we decided to initiate the analysis of this matter by undertaking the study of *GLT1* transcriptional regulation in *S. cerevisiae*.

In the present work, we prepared *GLT1-lacZ* fusions which allowed the study of *GLT1* expression in *GDH1* and *gdh1* strains in the presence of various nitrogen sources. A collection of 5' deletion derivatives of the *GLT1* promoter was prepared in order to determine the DNA sequences that could be involved in transcriptional regulation. We also studied the role of three transcriptional activators (Gcn4p, Gln3p, Gat1p/Nil1p) (5, 11, 22, 34, 41) and of a repressor protein (Uga43p/Dal80p) (12, 15) on *GLT1* expression, all of which have been shown to be involved in regulation of expression of genes coding for enzymes of amino acid biosynthesis or of nitrogen catabolism.

Our results indicate that: first, under conditions of glutamate excess, *GLT1* expression is governed by both, glutamate mediated-repression and Gln3p, Gcn4p-mediated activation; second, under derepressive conditions, *GLT1* expression could be positively regulated by a Zn_2-Cys_6 binuclear cluster factor, Gcn4p, Gln3p and by an imperfect poly (dA-dT) promoter element;

and third, under amino acid deprivation, *GLT1* expression is moderatively regulated by Gcn4p.

MATERIALS AND METHODS

Strains. Table 1 describes the characteristics of the strains used in this study. Null mutants impaired in *GCN4*, *GLN3* or *GAT1* were derived from strain CLA1 by gene replacement using the 3.7 Kb *Bst*II-*Mlu*I restriction fragment of pM214 (21), the *Aat*II digested pPM62 (34) or plasmid pRR336 previously digested with *Xba*I-*Eco*RI (11), thus obtaining CLA100, CLA101 and CLA102. MAR1 was obtained by *GDH1* gene disruption with pLV3 linearized with *Bgl*II (2).

Growth conditions. Strains were routinely grown on minimal medium (MM) containing salts, trace elements and vitamins following the formula of yeast nitrogen base (Difco). Filter sterilized glucose (2%) was used as the carbon source and 0.2% $(NH_4)_2SO_4$, 0.1% glutamate, glutamine, asparagine or proline were used as nitrogen sources. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (w/v). Cells were incubated at 30°C under shaking (250 rpm). For amino acid deprivation experiments, CLA1/pLOU1 or its *gcn4Δ*/pLOU1 derivative were inoculated into 10 ml of YPD, incubated at 30°C with shaking for 6 h, washed twice, and resuspended in MM. An aliquot was inoculated into 100 ml of MM to give OD_{600} of 0.05. This culture was incubated at 30°C with shaking for 6

h, harvested, resuspended in 10 ml of MM, and inoculated into 100 ml of MM to give OD_{600} of 0.2 and into 100 ml of MM + 10 mM 3-aminotriazole (3-AT) to give OD_{600} of 0.5. After 6 h of incubation at 30°C with shaking (250 rpm), cultures were centrifuged and used for β-Gal determinations.

Determination of GOGAT and β-galactosidase (β-Gal) activities. Yeast total extracts were prepared from cultures inoculated at an OD_{600} of 0.05 and harvested at an OD_{600} between 0.8-1.0. Cells were washed twice with H_2O and once with the corresponding extraction buffer (13, 37). The pellet was stored at -20°C until used. Soluble extracts were prepared by suspending whole cells in their corresponding extraction buffer and grinding them with glass beads in a Vortex mixer. Yeast GOGAT (EC 1. 4. 7. 1) activity was determined by the method described by Cogoni et al. (12). Specific activity was expressed as nanomoles of NADH oxidized per minute per milligram of protein. β-galactosidase activities were determined by the method described by Rose and Botstein (37). β-Gal specific activity was expressed as nanomoles of o-nitrophenol produced per minute per milligram of protein. Protein was measured by the method of Lowry et al. (29), with bovine serum albumin as a standard.

Construction of *lacZ* fusions. Plasmid Yc14 previously described and sequenced (13, 17), contains 2 Kb of the *GLT1* coding sequence, the full *GLT1* promoter and 30 bp of the *UGA3* coding sequence. Yc14 DNA was digested with *Eco*RI and used as template for PCR amplification. Deoxyoligonucleotide F1, contained a *Bam*HI site and 18 bp of the *UGA3* coding region (5'-CGCGCGGGATCCCAATTTCAGCTTCTCCAC-3').

Deoxyoligonucleotide R1, contained a *Sal*I site, 8 bp upstream the *GLT1* coding region and 3 bp downstream the *GLT1* promoter region (5'-GCGCGCGGTCGACACTGGCATGCT-3'). F1 an R1 deoxyoligonucleotides were used to amplify the complete *GLT1* promoter. In order to obtain a 5' *GLT1* promoter deletion family, the pertinent forward deoxyoligonucleotides were designed based on the *GLT1* promoter sequence. Deoxyoligonucleotide R1 was also used to amplify the full promoter and the 18 individual deletions. The entire family of PCR products was fused in frame to the *E. coli lacZ* gene of YEp363 (2μm *LEU2*) (35), generating 19 fusion plasmids, numbered from pLOU1 to pLOU19. The PCR product carrying the full *GLT1* promoter was also fused in frame to the *E.coli lacZ* gene of YEp353 (2μm *URA3*) (35), generating plasmid pSIM1. All fusion plasmids were sequenced with an automated Applied Biosystems 373 DNA sequencer (W. M. Keck Foundation, Yale University).

Yeast transformation. *S. cerevisiae* was transformed by the method described by Ito et al. (26). To generate null derivatives, transformants were selected for uracil prototrophy on MM supplemented with auxotrophic requirements as needed. Pertinent strains were transformed with the *lacZ* fusion plasmids or, when appropriate, with YEp363. Transformants were selected for either leucine or uracil prototrophy on MM supplemented with auxotrophic requirements as needed.

Primer extension RNA analysis. Primer extension reactions were performed by standard procedures (38). In order to determine chromosomal *GLT1* transcription initiation sites, total RNA was isolated from strain CLA1 grown on MM with 0.2% $(NH_4)_2SO_4$ as nitrogen source. A

deoxyoligonucleotide containing the first 21 nucleotides of the *GLT1* coding region was prepared and used in the primer extension reactions. The transcription initiation sites present in the different *lacZ* fusion constructs, were also determined. Primer extension reactions were carried out with total RNA extracted from the pertinent strains grown on MM with 0.2% $(NH_4)_2SO_4$ as nitrogen source, and a deoxyoligonucleotide containing 23 nucleotides of the *lacZ* coding region.

RESULTS AND DISCUSSION

Sequence analysis of *GLT1* promoter region and determination of transcription initiation sites. As stated in the Introduction, the role of GOGAT in glutamate biosynthesis and its regulation have not been studied in yeast. In order to address this matter, we analyzed the nucleotide sequence located upstream of the *GLT1* coding region, which was contained in the previously reported plasmid Yc14 (13). As Fig. 1 shows, *GLT1* was located in opposite orientation, next to the *UGA3* gene which codes for a transcriptional activator of the genes involved in gamma-aminobutyrate catabolism (1). Intragenic sequences may act as sites for trans-acting regulatory elements of either of the two divergent genes, *GLT1* and *UGA3*. Such sequences could face or partly overlap sites with the opposite orientation in the complementary DNA strand that regulate the alternative divergent gene. One could expect that simple occupancy of either sequence by its cognate high affinity regulator may

interfere with regulation of the alternative divergent gene. The study of *UGA3* expression may help define this matter.

Most of the genes encoding amino acid biosynthetic enzymes in *Saccharomyces cerevisiae* are subject to a cross-pathway regulatory system known as the general amino acid control that stimulates their expression under conditions of amino acid starvation. Gcn4p is the direct positive regulator of gene expression in this system (22). Examination of the *GLT1* promoter revealed a canonical Gcn4p binding site ATGACTC ($GCN4^{(1)}$) (Fig. 1) located between positions -477 and -466. *GLT1* promoter also carries four non-canonical binding sites (Fig. 1) with low affinity for Gcn4p (31, 44), TGCGTA from -399 to -393 ($GCN4^{(2)}$), TTAGTCAT from -267 to -260 ($GCN4^{(3)}$); ATTAATCA from -193 to -186 ($GCN4^{(4)}$); and GTGATTAAC from -43 to -35 ($GCN4^{(5)}$).

The *GLT1* promoter also contained three GATAA sequences GATAAG from -377 to -372 ($GATA^{(1)}$), CTTATC (complementary, GATAAG) from -238 to -233 ($GATA^{(2)}$) and GATAAC from -168 to -164 ($GATA^{(3)}$) (Fig. 1), which can constitute the cis-acting element, UAS_{NTR} (41). UAS_{NTR} has been proposed as a binding site for two transcriptional activators, Gln3p and Gat1p/Nil1p (4, 11, 34, 42), which regulate the expression of nitrogen modulated genes.

Downregulation of nitrogen controled gene expression is accomplished by the action of the GATA family member Dal80p/Uga43p. The Dal80p binding site, URS_{GATA}, consists of a pair of GATA-containing sequences

oriented tail-to-tail or head-to-tail (15). As can be seen in Fig. 1, the *GLT1* promoter harbors two of the above mentioned GATA sequences oriented tail-to-tail; these could constitute a Dal80p binding site, although the intervening distance between them (63bp) is larger than that previously reported (15 to 35 bp) (15).

At least 79 fungal transcription activating factors containing a six-cysteine-two zinc DNA binding domain called Zn_2-Cys_6 binuclear cluster have been found (39). DNA targets for several members of this family of proteins have two inverted CCG half-sites separated by a spacing characteristic of the particular protein that recognizes it (27). The two inverted CGG half sites separated by 15 base pairs present in the *GLT1* promoter (Fig. 1) could also constitute a binding site for members of the Zn_2-Cys_6 binuclear cluster family of proteins.

Many yeast promoters contain homopolymeric (dA-dT) sequences (43). Analysis of the function of these sequences in transcriptional activation has suggested that perfectly homopolymeric sequences function by virtue of their intrinsic structure. For imperfect poly (dA-dT) tracts, it has been proposed that its transcriptional effects might be mediated in part or completely by specific DNA-binding proteins (47). *GLT1* promoter also bears two poly (dA-dT) sequences: the first one composed of a16 poly (dA-dT) tract with two imperfections located from position -292 to position -276 [poly(dA-dT)^1 in Fig. 1]; the second one consisted of a 19 poly (dA:dT) tract with two imperfections located from -2 to +17 [poly(dA-dT)^2 in Fig. 1].

Primer extension analysis, (Fig. 2) defined two transcription initiation sites in *GLT1*, which are shown in Fig. 1 at positions +1 and +52. The results presented in Fig. 2, indicate that the +1 initiation site is stronger than the +52 site. Two putative TATA boxes differing from the TATAAA canonical sequence, were also found (Fig. 1). Either TATACTA or TATTTA sequences can substitute TATAAA in transcription initiation (19). Constructions from pLOU14 to 17 and on were only able to initiate transcription from +52. It could be possible that each one of these initiation sites together with $TATA^{(1)}$ or $TATA^{(2)}$ could signal transcription under different physiological conditions. If this were the case, the first element would direct transcription regulated by glutamate mediated repression and by Gcn4p, Gln3p, and the putative Zn_2-Cys_6 binuclear cluster mediated activation. The second element would direct transcription mediated by the poly (dA-dT) element by itself or in combination with a glutamate-sensitive activator.

***GLT1* expression was repressed in the presence of glutamate, the remaining expression was *GLN3* and *GCN4*-dependent.** It has been previously observed that mutants impaired in *GDH1* display increased GOGAT activity (2), suggesting that *GLT1* could be negatively modulated by glutamate and that in a *gdh1* mutant, glutamate limitation could result in *GLT1* derepression. In order to determine whether *GLT1* expression was regulated by the nature of the nitrogen source, we determined GOGAT and β-Gal activities in a wild type strain and in a *gdh1* mutant. Both strains harbored either plasmid pLOU1 containing

GLT1 promoter fused to the complete β-Gal coding region, or the vector YEp363 (see Materials and Methods). As expected, in the presence of YEp363, no β-Gal activity was detected, and GOGAT activity values were similar to those found in the presence of pLOU1 (Table 2). As Table 2 and Fig. 3 (row 1) show, GOGAT and β-Gal activities were higher in the *gdh1* mutant strain when grown on ammonium or proline as sole nitrogen sources, than those found in the wild type strain grown under similar conditions. In the presence of glutamate, glutamine or asparagine, both GOGAT and β-galactosidase activities decreased and achieved similar values in extracts obtained from either the wild type or the *gdh1* strains (Table 2). These results indicate that *GLT1* expression was repressed in the presence of glutamate rich nitrogen sources.

To analyze whether Gln3p, Gat1p/Nil1p, Gcn4p or Dal80p/Uga43p had a role in *GLT1* expression, plasmid pLOU1, was transformed into *gcn4Δ*, *gln3Δ*, *gat1Δ* or *uga43Δ* mutant strains, and β-Gal activity was determined (Table 1 and Fig. 4 row 1). It was found that with ammonium or proline as nitrogen sources, the lack of Gln3p severely diminished β-Gal activity; impairment of Gcn4p had a slight effect on this activity, while the lack of Gat1p, had no effect. Extracts obtained from cultures of the *gln3Δ*, and *gcn4Δ* showed decreased β-Gal activity as compared to extracts obtained from the wild type when either strain was, grown on glutamate, glutamine or asparagine. These results suggested that *GLT1* transcription of yeast cells grown in glutamate rich nitrogen sources was down-regulated by glutamate repression and up-regulated by Gln3p, and Gcn4p transcriptional activators. The capacity of Gln3p and Gat1p to activate

transcription appears to be nitrogen regulated, in such a way that *GLN3* stimulates transcription on glutamate and proline, and *GAT1* on ammonium and urea (42). However, neither Gln3p or Gat1p promote the expression of nitrogen-regulated genes on glutamine (42). Our results indicate, on one hand, that *GLT1* is not regulated by *GAT1*, and on the other, that *GLN3* activates *GLT1* expression in all nitrogen sources tested, including glutamine. It could be speculated that the *GLT1* promoter could have a higher affinity for the *GLN3* inactive form that has been postulated to be present in glutamine (5). Also as has been observed in other cases (36), *GLN3* could be acting in combination with the positive activator that should bind the CGG palindrome. Maybe in the case of *GLT1* expression, a less active form of *GLN3* could play an important role on glutamine when assisted by another activator.

Isogenic strains carrying the wild type *UGA43/DAL80* gene or the null allele were also transformed with pLOU1. As Table 2 shows, lack of Uga43p/Dal80p did not result in derepressed *GLT1* expression in the presence of glutamate, indicating that glutamate mediated repression was not *UGA43/DAL80* dependent. Further experiments will be required to determine the nature of the cis and trans-acting elements which mediate glutamate repression.

In order to address if *GLT1* expression was regulated during amino acid deprivation by the general amino acid control mediated by Gcn4p (22), β-Gal was determined in extracts from cultures of the wild type strain and of the *gcn4Δ* mutant grown in the presence and absence of 3-aminotriazole (3-AT), a competitive inhibitor of His3p. In the presence of this analogue

cells become deprived for histidine. β-Gal activity was two-fold higher in extracts obtained from the wild type strain grown in the presence of 3-AT, as compared to that found in its absence (1280 VS 2170 nmol.min^{-1}.mg^{-1}). This increment was not observed in the *gcn4Δ* mutant strain (600 VS 550 nmol.min^{-1}.mg^{-1}). These results indicate that *GLT1* expression was increased during amino acid deprivation and that this increase was Gcn4p-dependent (22). Since glutamate is precursor in the biosynthesis of most amino acids, it may be expected that the genes coding for the enzymes involved in its biosynthesis would have to be responsive to starvation of a number of amino acids. However our results indicate that GOGAT (*GLT1*) is not strongly regulated by Gcn4p. The analysis of whether *GDH1* or *GDH3* transcription responds to amino acid limitation, will be very useful to fully understand the pathway(s) through which glutamate biosynthesis could be increased during amino acid starvation. The exact binding site(s) for Gcn4p on *GLT1* promoter remains to be determined. However our 5' deletion analysis suggests that the canonical *GCN4* binding site (*GCN4*(1)), plays no role in *GLT1 GCN4*-dependent transcriptional activation, since when it is deleted (pLOU3), *GLT1* transcription is not decreased. It is clear that pLOU4 dependent β-Gal activity is decreased in a null *gcn4Δ* derivative, indicating that the *GCN4* binding site (*GCN4*(2)) could play a more important role than (*GCN4*(1)) in *GCN4*-mediated transcriptional activation. It could also be speculated that the *GCN4*(3) putative binding site could play a role in *GLT1* gene activation together

with the poly (dA-dT)(1), since it has been suggested that during gene activation of promoters harboring both, a poly (dA-dT) tract and a *GCN4* binding site, transcription could be either hindered or promoted through chromatin reorganization (47).

Deletion analysis of *GLT1* promoter. A collection of 5' deletions of increasing size affecting the *GLT1* promoter was prepared as described in Material and Methods. As Fig. 3 row 4 shows, when a *GDH1* strain harbouring pLOU4, which lacks the most 5' 173 bp of the *GLT1* promoter was grown on ammonium, β-Gal activity was slightly increased, as compared to the value obtained with the *GDH1* strain carrying pLOU1. This increment was more evident when β-Gal was determined in a *gdh1* strain carrying pLOU4, which showed a nearly threefold higher β-Gal activity as compared to that found in the *gdh1* strain carrying pLOU1. These results suggested that pLOU4 had lost a target for negative regulation (upstream repressing region, URR1) (Fig. 3). Deletions covering bp from -608 to -413; from -608 to -395; from -608 to -381 and from -608 to -373 (pLOU5, 6, 7 and 8) resulted in decreased β-Gal activity in both *GDH1* and *gdh1* strains, indicating that this region could contain DNA binding sites for transcriptional activators. As Fig. 1 shows, this region contained putative binding sites for Gcn4p, Gln3p and for a Zn_2-Cys_6 binuclear cluster activator. In order to determine whether the observed increase in β-Gal activity, fostered by pLOU4, was *GCN4*, *GLN3* or *GAT1/NIL1* dependent, pertinent strains were transformed with this plasmid. As Fig. 4 (row 2) shows, increased β-Gal activity was mainly *GLN3*

and *GCN4* dependent. Fig. 3 also shows that increased β-Gal activity was still glutamate sensitive indicating that pLOU4 had retained a cis-acting region able to respond to glutamate. Further deletions (pLOU 9 to 14) resulted in a constant increase of β-Gal activity in the *gdh1* derivatives, but practically no changes in β-Gal activity of the corresponding *GDH1* strains. Deletions present in pLOU15 to 17 resulted in a clear increase of β-gal activity in both *GDH1* and *gdh1* strains, the highest activity being observed after removal of the first 573 bp (pLOU17). β-Gal activity of constructions pLOU1 to 13 was clearly diminished by the presence of glutamate in the medium, however when β-Gal was determined in strains harbouring constructions from pLOU14 to 17, although addition of glutamate to the medium reduced β-Gal activity, the values were several-fold higher than those found with the full promoter in cells grown in the presence of glutamate. These results suggested, on one side, that a glutamate responsive negative acting region (upstream repressing region URR2) was localized from -373 to -119; and on the other, that the region contained between -35 and +40 contained a target for a trans-acting positive regulatory element. As Fig. 3 shows, this DNA segment contained a poly (dA-dT) tract, which has been considered as a promoter element able to stimulate transcription (47). In the presence of glutamate, transcription fostered by pLOU17 is diminished, although the β-Gal levels determined in this condition are threefold higher than those found under repressive conditions (MAR1/pLOU1 on glutamate). Thus it is possible to consider that the so far undetermined activator, which we propose acts in combination with the poly (dA-dT) tract, could be glutamate inactivated.

β-Gal activity fostered by pLOU16 and 17 was also found in *gln3Δ*, *gat1Δ* or *gcn4Δ* null derivatives (Fig. 4), indicating that the poly (dA-dT) tract acted either independently of activators or that it was assisted by an as yet unrecognized positive regulatory element. This analysis suggested that *GLT1* transcriptional regulation depended on the action of both, negative regulatory regions (URR1 and URR2) and positive acting elements. Both URR regions could be targets for glutamate-mediated repression since when they were removed, *GLT1* expression was no longer fully repressed by glutamate. In addition, our results also indicate that, from the putative cis-acting sites depicted in Fig. 1, the following could have a positive role in *GLT1* transcription: a) the *GCN4* (2) binding site from -396 to -390; b) the *GLN3* binding site from -377 to -372 (GATA(1)); c) the CCG palindromic region located from -412 to -388; and d) the poly (dA-dT) tract located from -2 to +17. No β-Gal activity was determined in strains carrying constructions present in pLOU18 and pLOU19, indicating that the promoter fragment contained from +40 to +100 was unable to initiate *GLT1* transcription.

In regard to the role of GOGAT in glutamate biosynthesis, our results indicate that: i) under low glutamate conditions *GLT1* transcription is considerably low, so it could be suggested that GOGAT could have an important role in glutamate biosynthesis under conditions where this amino acid becomes limiting and ii) GOGAT could constitute an ancillary pathway furnishing a low but sustained glutamate production, even in the presence of $NADP^+$-GDH i.e., in the presence of a relatively high glutamate

pool. Suggesting that a high intracellular glutamate pool could be needed for optimal growth. Since it has been reported that null GOGAT mutants grow as well as the wild type strain on ammonium (2), the high glutamate need could be restricted to certain physiological conditions, like high external osmolality (14) or during sporulation, since in this condition, both carbon and nitrogen are limiting and this could result in glutamate deprivation.

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FIGURE LEGENDS

FIG. 1. *GLT1* promoter sequence. Putative Gcn4p (*GCN4*), Gln3p, Gat1p binding sites (GATA), CGG-N_{15}-CCG palindrome, and poly (dA-dT) regions are boxed and numbered independently and progresively starting from the most 5'. Two putative TATA boxes (TATA 1 and TATA 2) are indicated; as well as the two transcription initiation sites, at positions +1 and +52. The 714 bp fragment represented in this figure, includes a 30 bp sequence of the *UGA3* coding region. *Bam*H1 and *Sal*1 sites were added and used to clone this fragment into the 2μm *LEU2* *lacZ* vector YEp363 generating plasmid pLOU1.

FIG. 2. Primer extension analysis. A. Transcription initiation sites (lane PE) of the *GLT1* gene, carried out with total RNA obtained from the wild type strain CLA1. B Representative results of primer extension analysis carried out with total RNA obtained from: lane 1, CLA1 strain transformed with anyone of plasmids pLOU1, 3, 4, 6. 8, 9, 10, or 11); lane 2, CLA1 strain transformed with anyone of plasmids from pLOU14 to 17. Sequence ladder was produced with the same deoxyoligonucleotide used for the primer extension reaction which was described in Materials and Methods.

FIG. 3. β-galactosidase activities of 5' deletions of the *GLT1* promoter. *GLT1* full promoter and 5' deletions were cloned into the 2μm *LEU2* *lacZ* vector YEp363, generating plasmids from pLOU1 to 19. These plasmids were transformed into either the CLA1 *GDH1* wild type strain or the MAR1 *gdh1* mutant strain. The 5' region carried in each plasmid is indicated in rows from 1 to 19. β-Gal activity was determined in extracts obtained from

cells grown on either 0.2% ammonium sulfate or 0.1 % glutamate. ND, not determined.

Gcn4p putative binding sites ( ); palindrome (); Gln3p putative binding sites ( ); poly (dA-dT) ( ); putative TATA boxes ( ); transcription initiation sites ( ). Putative upstream repressive regions (URR).

FIG. 4. Effect of *gln3*, *gat1* and *gcn4* null mutations on β-galactosidase activity in 5' deletions of the *GLT1* promoter. Mutant strains, CLA101 (*gln3Δ*), CLA102 (*gat1Δ*) and CLA100 (*gcn4Δ*) harbouring plasmids pLOU1, pLOU4, pLOU13, pLOU16 or pLOU17 (lines 1, 4, 13, 16, and 17) were grown on 0.2 % ammonium sulfate as nitrogen source, and β-Gal activity was determined. The reported β-Gal activities are averages of values obtained in three independent experiments. *GLT1* promoter regions are represented as in Fig. 3. Variations were <15 %.

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source
CLA1	*MATa GDH1 GDH3 GLT1 ura 3 leu 2*	(2)
CLA1-0	*MATa GDH1 GDH3 GLT1 ura 3 leu 2* / YEp363 *(2μm LEU2)*	this study
CLA1-1	*MATa GDH1 GDH3 GLT1 ura 3 leu 2* / pLOU1(*GLT1-lacZ* 2μm *LEU2*)	this study
CLA-100	*MATa GDH1 GDH3 GLT1 gcn4Δ ::URA3 leu2* / pLOU1(*GLT1-lacZ* 2μm *LEU2*)	this study
CLA-101	*MATa GDH1 GDH3 GLT1 gln3Δ ::URA3 leu2* / pLOU1(*GLT1-lacZ* 2μm *LEU2*)	this study
CLA-102	*MATa GDH1 GDH3 GLT1 gat1Δ ::URA3 leu2* / pLOU1(*GLT1-lacZ* 2μm *LEU2*)	this study
MAR1	*gdh1Δ::URA3 GDH3 GLT1 leu2*	this study
MAR1-0	*gdh1Δ::URA3 GDH3 GLT1 leu2* / YEp363 (2μm *LEU2)*	this study
MAR1-1	*gdh1Δ::URA3 GDH3 GLT1 leu2* / pLOU1 (*GLT1-lacZ* 2μm *LEU2*)	this study
27034b	*MATa GDH1 GDH3 GLT1 UGA43 ura3 leu 2* / pSIM1 *(GLT1-lacZ 2μm URA3)*	(13)
30078c	*MATa GDH1 GDH3 GLT1 uga43Δ ura3* / pSIM1 *(GLT1-lacZ 2μm URA3)*	(13)

TABLE 2. β-galactosidase and GOGAT specific activities[a]

	Strain (relevant genotype)																	
	CLA1-0 *GDH1*/YEp363		CLA1-1 *GDH1*/pLOU1		MAR1-0 *gdh1Δ*/YEp363		MAR1-1 *gdh1Δ*/pLOU1		CLA100 *GDH1 gcn4Δ*/ pLOU1	CLA101 *GDH1 gln3Δ*/ pLOU1	CLA102 *GDH1 gat1Δ*/ pLOU1	27034b *GDH1 UGA43*/ pSIM1	30078c *GDH1 uga43Δ*/ pSIM1
	Sp act of:												
Nitrogen source	β-Gal	GOGAT	β-Gal	GOGAT	β-Gal	GOGAT	β-Gal	GOGAT	β-Gal	β-Gal	β-Gal	β-Gal	β-Gal
Ammonium	ND	40	1280	41	ND	71	3650	69	600	230	1150	850	630
Proline	ND	40	1580	41	ND	53	2360	55	600	290	1250	3850	4250
Glutamate	ND	22	550	24	ND	24	600	25	200	270	392	910	750
Glutamine	ND	22	520	21	ND	27	710	29	200	60	362	410	340
Asparagine	ND	23	450	25	ND	23	450	24	250	100	308	1340	1000

[a]The reported values are expressed in nmol.min^{-1}.mg^{-1}, and represent the mean of three independent experiments. Variations were ≤ 15%.
[b]ND, not detected

TABLE 3. β-galactosidase specific activities[a]

	Strain (relevant genotype)	
	CLA1-1 *GDH1*/pLOU1	CLA100 *GDH1 gcn4Δ* /pLOU1
	β-galactosidase ($nmol.min^{-1}.mg^{-1}$)	
Growth conditions		
Ammonium	1280	600
Ammonium + 10 mM 3-AT	2170	550

[a]The reported values are averages of the mean of three experiments, variations were ≤ 15%.

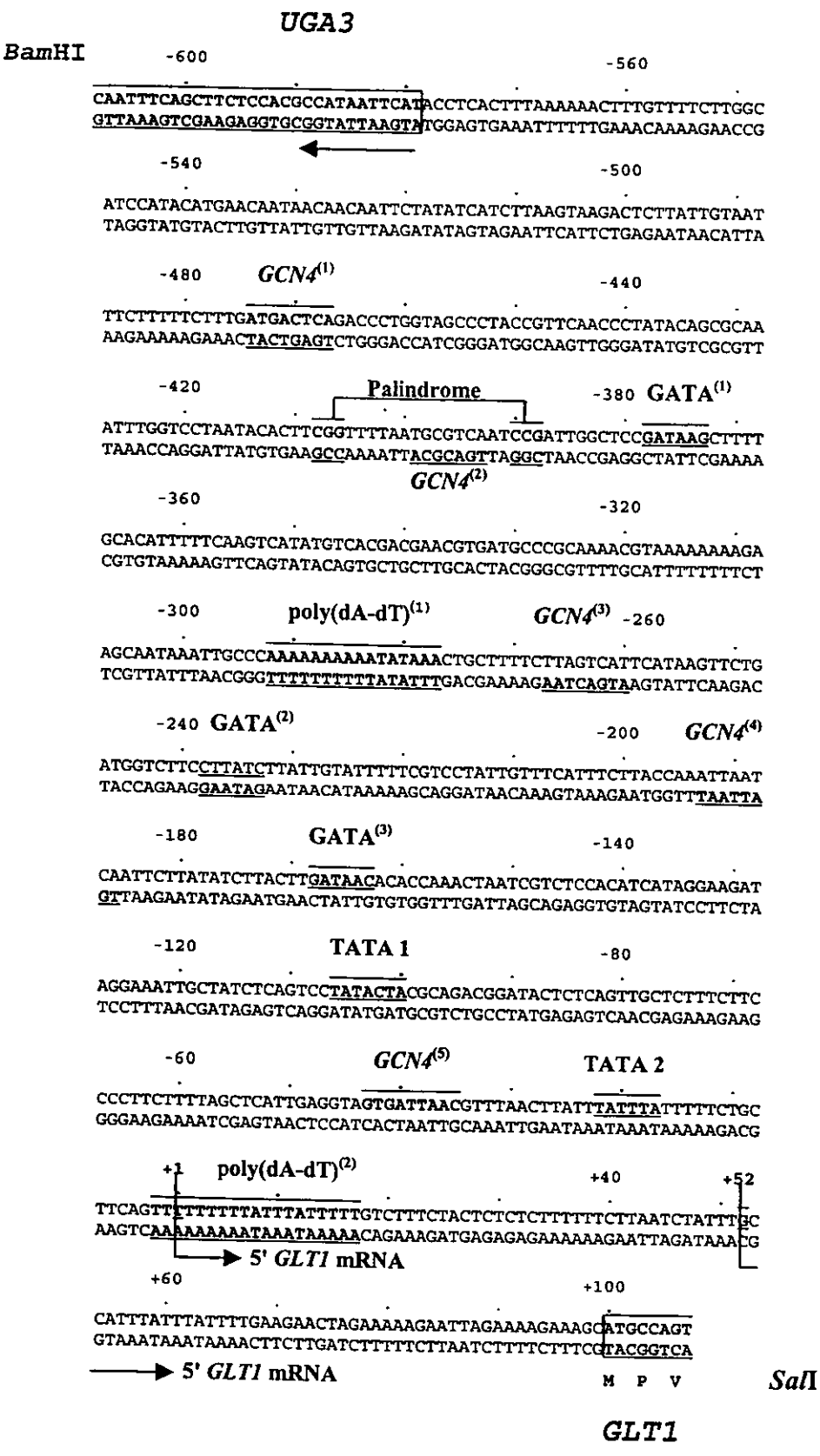

UGA3
BamHI
-600
-560
CAATTTCAGCTTCTCCACGCCATAATTCATACCTCACTTTAAAAAACTTTGTTTTCTTGGC
GTTAAAGTCGAAGAGGTGCGGTATTAAGTATGGAGTGAAATTTTTTGAAACAAAAGAACCG
-540
-500
ATCCATACATGAACAATAACAACAATTCTATATCATCTTAAGTAAGACTCTTATTGTAAT
TAGGTATGTACTTGTTATTGTTGTTAAGATATAGTAGAATTCATTCTGAGAATAACATTA
-480
GCN4(1)
-440
TTCTTTTTCTTTGATGACTCAGACCCTGGTAGCCCTACCGTTCAACCCTATACAGCGCAA
AAGAAAAAGAAACTACTGAGTCTGGGACCATCGGGATGGCAAGTTGGGATATGTCGCGTT
-420
Palindrome
-380
GATA(1)
ATTTGGTCCTAATACACTTCGGTTTTAATGCGTCAATCCGATTGGCTCCGATAAGCTTTT
TAAACCAGGATTATGTGAAGCCAAAATTACGCAGTTAGGCTAACCGAGGCTATTCGAAAA
GCN4(2)
-360
-320
GCACATTTTTCAAGTCATATGTCACGACGAACGTGATGCCCGCAAAACGTAAAAAAAAGA
CGTGTAAAAAGTTCAGTATACAGTGCTGCTTGCACTACGGGCGTTTTGCATTTTTTTTCT
-300
poly(dA-dT)(1)
GCN4(3)
-260
AGCAATAAATTGCCCAAAAAAAAAATATAAACTGCTTTTCTTAGTCATTCATAAGTTCTG
TCGTTATTTAACGGGTTTTTTTTTTATATTTGACGAAAAGAATCAGTAAGTATTCAAGAC
-240
GATA(2)
-200
GCN4(4)
ATGGTCTTCCTTATCTTATTGTATTTTTCGTCCTATTGTTTCATTTCTTACCAAATTAAT
TACCAGAAGGAATAGAATAACATAAAAAGCAGGATAACAAAGTAAAGAATGGTTTAATTA
-180
GATA(3)
-140
CAATTCTTATATCTTACTTGATAACACACCAAACTAATCGTCTCCACATCATAGGAAGAT
GTTAAGAATATAGAATGAACTATTGTGTGGTTTGATTAGCAGAGGTGTAGTATCCTTCTA
-120
TATA 1
-80
AGGAAATTGCTATCTCAGTCCTATACTACGCAGACGGATACTCTCAGTTGCTCTTTCTTC
TCCTTTAACGATAGAGTCAGGATATGATGCGTCTGCCTATGAGAGTCAACGAGAAAGAAG
-60
GCN4(5)
TATA 2
CCCTTCTTTTAGCTCATTGAGGTAGTGATTAACGTTTAACTTATTTATTTATTTTTCTGC
GGGAAGAAAATCGAGTAACTCCATCACTAATTGCAAATTGAATAAATAAATAAAAAGACG
+1
poly(dA-dT)(2)
+40
+52
TTCAGTTTTTTTTTTATTTATTTTTGTCTTTCTACTCTCTCTTTTTTCTTAATCTATTTGC
AAGTCAAAAAAAAAATAAATAAAAACAGAAAGATGAGAGAGAAAAAAGAATTAGATAAACG
5' GLT1 mRNA
+60
+100
CATTTATTTATTTTGAAGAACTAGAAAAAGAATTAGAAAAGAAAGCATGCCAGT
GTAAATAAATAAAACTTCTTGATCTTTTTCTTAATCTTTTCTTTCGTACGGTCA
5' GLT1 mRNA
M P V
SalI
GLT1

FIG. 1

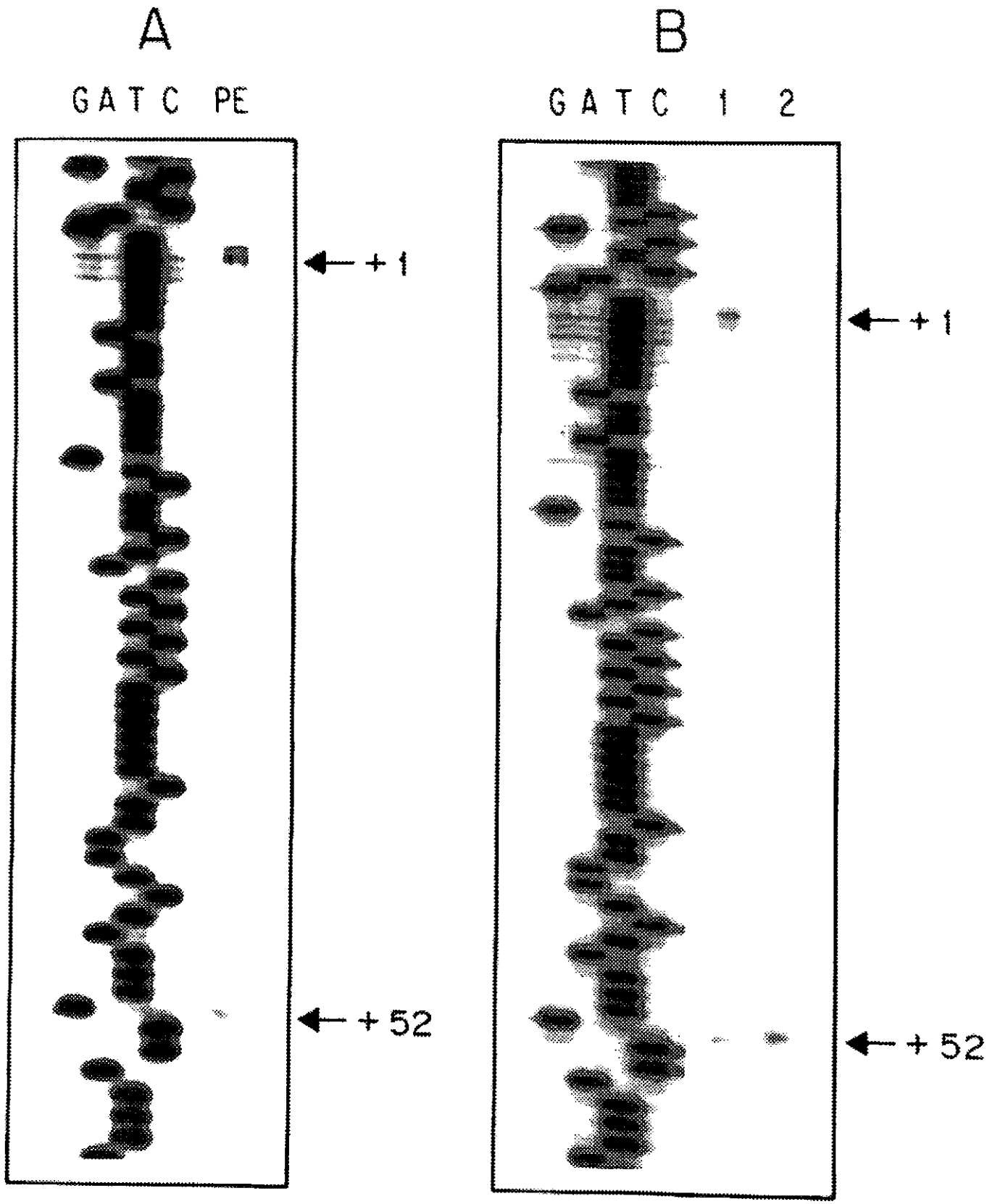
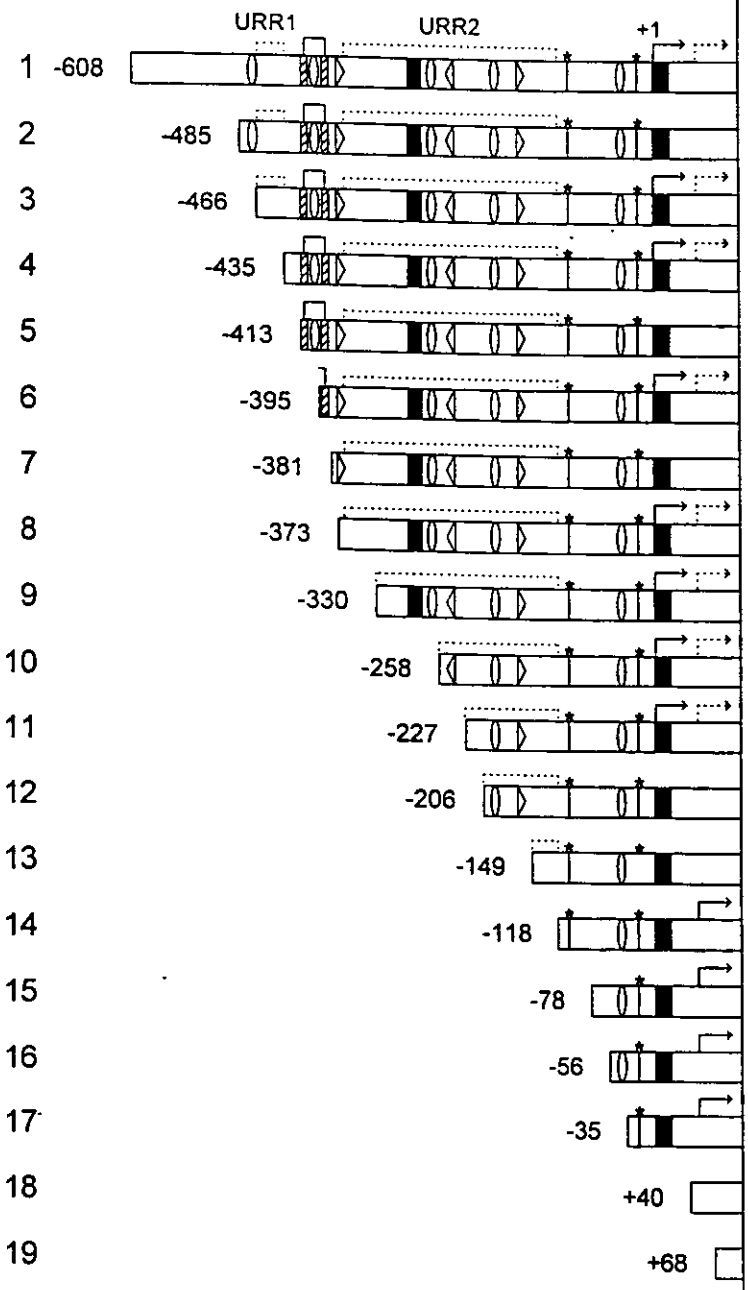


FIG. 2



		ß-galactosidase (nmol.min^{-1}.mg^{-1})			
		CLA1 *GDH1*		MAR1 *gdh1Δ*	
		ammonium	glutamate	ammonium	glutamate
1	-608	1280	550	3650	600
2	-485	1000	440	4780	890
3	-466	1250	600	3530	540
4	-435	1570	780	9500	1200
5	-413	1410	800	7410	930
6	-395	710	310	4020	710
7	-381	740	400	3860	660
8	-373	660	330	2250	570
9	-330	570	410	4130	540
10	-258	750	620	5020	840
11	-227	560	700	5060	1000
12	-206	1230	1050	5400	1220
13	-149	920	670	7110	1110
14	-118	1440	770	8250	1470
15	-78	2110	1800	8100	1610
16	-56	2320	1990	8530	1780
17	-35	4080	3110	9400	1860
18	+40	80	40	40	10
19	+68	ND	ND	ND	ND

FIG. 3

ß-galactosidase
(nmol·min^{-1}·mg^{-1})
CLA1 WT
CLA101 gln3Δ
CLA102 gat1Δ
CLA100 gcn4Δ
URR1
URR2
1
4
13
16
17
-608
-435
-149
-56
-35
1280
230
1150
600
1570
870
2110
530
920
1490
1030
890
2320
3290
2950
3750
4080
6180
4370
4290

FIG. 4

Capítulo V

GDH3 encodes a glutamate dehydrogenase isozyme, a previously unrecognized route for glutamate biosynthesis in *Saccharomyces cerevisiae.*

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GDH3 Encodes a Glutamate Dehydrogenase Isozyme, a Previously Unrecognized Route for Glutamate Biosynthesis in *Saccharomyces cerevisiae*

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It has been considered that the yeast *Saccharomyces cerevisiae*, like many other microorganisms, synthesizes glutamate through the action of $NADP^+$-glutamate dehydrogenase ($NADP^+$-GDH), encoded by *GDH1*, or through the combined action of glutamine synthetase and glutamate synthase (GOGAT), encoded by *GLN1* and *GLT1*, respectively. A double mutant of *S. cerevisiae* lacking $NADP^+$-GDH and GOGAT activities was constructed. This strain was able to grow on ammonium as the sole nitrogen source and thus to synthesize glutamate through an alternative pathway. A computer search for similarities between the *GDH1* nucleotide sequence and the complete yeast genome was carried out. In addition to identifying its cognate sequence at chromosome XIV, the search found that *GDH1* showed high identity with a previously recognized open reading frame (*GDH3*) of chromosome I. Triple mutants impaired in *GDH1*, *GLT1*, and *GDH3* were obtained. These were strict glutamate auxotrophs. Our results indicate that *GDH3* plays a significant physiological role, providing glutamate when *GDH1* and *GLT1* are impaired. This is the first example of a microorganism possessing three pathways for glutamate biosynthesis.

Two pathways for ammonium assimilation and glutamate biosynthesis have been found in a variety of organisms. The first one, described by Holzer and Schneider in 1957 (12), is mediated by $NADP^+$-glutamate dehydrogenase ($NADP^+$-GDH; EC 1.4.1.4), which catalyzes the reductive amination of 2-oxoglutarate to form glutamate. In an alternative pathway demonstrated by Tempest et al. (25), glutamate is aminated to form glutamine by glutamine synthetase (GS; EC 6.3.1.2), the amide group of which is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT; EC 1.4.1.13), resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The GS-GOGAT pathway has been found in several microorganisms (2, 13, 16, 23) and in higher plants (18).

In *Saccharomyces cerevisiae*, both pathways for glutamate biosynthesis are present (7, 19). Mutants altered in $NADP^+$-GDH have been isolated (6); these show a higher doubling time than that of the wild type when both strains are grown on minimal medium supplemented with ammonia as the sole nitrogen source. Mutants impaired in GOGAT activity were selected from $NADP^+$-GDH-less mutants as glutamate auxotrophs (7, 19). Genetic analysis of one of these mutants showed that the lack of GOGAT activity was due to the presence of two mutations (*gus1* and *gus2*), which suggested the existence of two GOGAT enzymes in *S. cerevisiae* (7). Cloning of the GOGAT structural gene (*GLT1*) and construction of null GOGAT mutants definitively established that this yeast possesses a single NADH-GOGAT enzyme (4) and that GOGAT-less mutants (7) which cannot be complemented with *GLT1* (unpublished results) are probably impaired in *GLT1* regulation. In this paper we report the characterization of strains impaired in either *GDH1*, *GLT1*, or both. Our results show that there is a third pathway for glutamate biosynthesis, mediated by an $NADP^+$-GDH encoded by *GDH3*.

Mutants impaired in *GDH1* or *GLT1* were derived from strains CLA1 (*ura3 leu2*), CLA2 (*ura3 LEU2*), and CLA3 (*URA3 leu2*) (Table 1). CLA4, a prototrophic derivative of CLA1, was used as a wild-type control (Table 1). Null *glt1*Δ mutants were obtained as previously described (4). Plasmids pLV1 and pLV2 harbored a 4.5-kb *GLT1* fragment and the *URA3* (pLV1) or *LEU2* (pLV2) yeast genes. Both plasmids were sequenced with the Sequenase V.2 kit (U.S. Biochemicals [USB]). Strains CLA2 and DAN1 were transformed according to the method described by Hinnen et al. (10) with *Bgl*II-linearized preparations of pLV1 and pLV2 yielding, respectively, strains CLA5 (*GDH1 GDH3 glt1*Δ::*URA3*) and DAN2 (*gdh1 GDH3 glt1*Δ::*LEU2*). Southern analysis was carried out as described by Sambrook et al. (22). The results indicated that plasmid insertion had occurred in the wild-type chromosomal *GLT1* gene. Isolation and characterization of an ethyl methanesulfonate (EMS)-derived *GDH1* mutant has been described previously (7). In order to obtain a null *GDH1* mutant, the following pair of deoxyoligonucleotides was prepared: A1, 5′CAGAATTTCAACAAGCTT3′, from bp 961 to bp 978 and A2, 5′ACCGATATCACCAGC3′, from bp 1410 to bp 1392 of the *GDH1* gene (20, 21). PCR amplification of yeast genomic DNA was carried out in a thermocycler (PTC-100; MJ Research, Inc.). The 450-bp amplification product was gel purified and ligated into the *Bam*HI-*Sal*I site of either YIp5 (24), harboring the *URA3* yeast gene, or YIp351 (9), harboring the *LEU3* yeast gene, generating plasmids pLV3 and pLV4. These subclones were sequenced by primer extension with the Sequenase V.2 kit (USB), confirming that we had specifically amplified a *GDH1* fragment. Plasmids pLV3 and pLV4 were linearized after *Bgl*II digestion and used to direct homologous recombination into the yeast genome, generating strain CLA6

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source
W303-1A	*MAT*a *ade1 leu2 ura3 trp1 his3 can1-100*	Yeast Genetic Stock Center
p49	*MAT*α *lys2 gal2*	Yeast Genetic Stock Center
CN31	*MAT*α *his1 gdh1*	Yeast Genetic Stock Center
CLA1	*MAT*a *GDH1 GDH3 GLT1 ura3 leu2* (W303-1A × p49)	This study
CLA2	*MAT*a *GDH1 GDH3 GLT1 ura3 LEU2*::YIp351	This study
CLA3	*MAT*a *GDH1 GDH3 GLT1 URA3*::YIp5 *leu2*	This study
CLA4	*MAT*a *GDH1 GDH3 GLT1 URA3*::YIp5 *LEU2*::YIp351	This study
CLA5	*MAT*a *GDH1 GDH3 glt1*Δ::*URA3 LEU2*::YIp351	This study
CLA6	*MAT*a *gdh1*Δ::*URA3 GDH3 GLT1 LEU2*::YIp351	This study
CLA7	*MAT*a *GDH1 gdh3*Δ::*LEU2 GLT1 URA3*::YIp5	This study
CLA8	*MAT*a *gdh1*Δ::*LEU2 GDH3 glt1*Δ::*URA3*	This study
CLA9	*MAT*a *GDH1 gdh3*Δ::*LEU2 glt1*Δ::*URA3*	This study
CLA10	*MAT*a *gdh1*Δ::*URA3 gdh3*Δ::*LEU2 GLT1*	This study
DAN1	*MAT*α *gdh1 GDH3 GLT1 ura3 leu2* (W303-1A × CN31)	This study
DAN2	*MAT*α *gdh1 GDH3 glt1*Δ::*LEU2 ura3*	This study
DAN3	*MAT*α *gdh1 gdh3*Δ::*URA3 glt1*Δ::*LEU2*	This study

(*gdh1*Δ *GDH3 GLT1*) from CLA2. Southern analysis was carried out with the 450-bp *GDH1* insert as a probe. The pattern of transformants clearly indicated that the insertion of the construct had occurred in the wild-type genomic sequence of *GDH1* (Fig. 1).

The *glt1*Δ mutant strain CLA5 (*GDH1 GDH3 glt1*Δ) showed no growth phenotype, so it was indistinguishable from a wild-type strain grown on ammonium (Fig. 2A). In all cases, cells were grown on minimal medium containing the same salts, trace elements, and vitamins used in yeast nitrogen base medium (Difco Laboratories, Detroit, Mich.). Glucose (2%) was used as the carbon source. As had been found previously (6, 7), *GDH1*-less mutants showed a twofold higher duplication time when grown on ammonium (Fig. 2A); on glutamate they grew as well as the wild-type strain (data not shown). A double null mutant devoid of *GDH1* and *GLT1* (CLA8; *gdh1*Δ *GDH3 glt1*Δ) was prepared by successively transforming strain CLA1 (*GDH1 GLT1 GDH3 ura3 leu2*) with plasmids pLV1 and pLV4. Unexpectedly, the CLA8 double mutant was able to grow on ammonium with a duplication time of 7 h (Fig. 2B), thus indicating the existence of a third pathway for glutamate biosynthesis. Similar results were obtained with a double mutant strain (DAN2) carrying the *GDH1*-less EMS-derived mutation and the *glt1*Δ null mutation (Fig. 2B).

Chromosome I sequencing revealed the presence of a

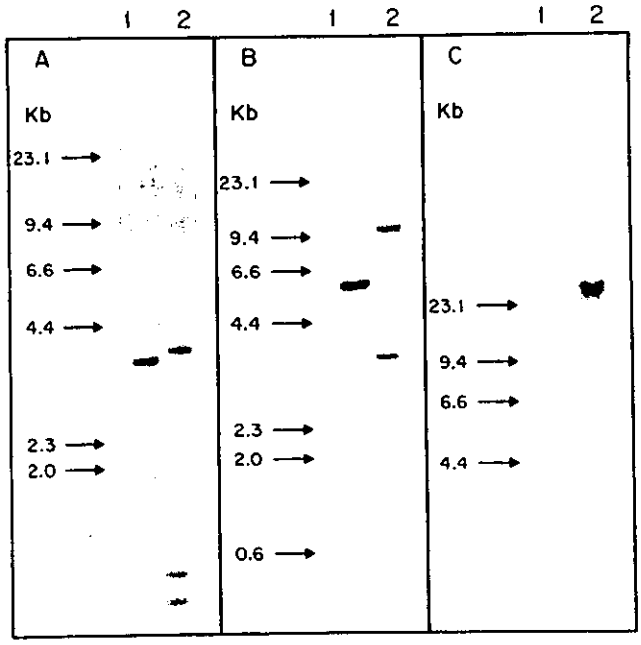

FIG. 1. Southern analysis of DNA. (A) Lanes: 1, wild-type strain (CLA4); 2, *gdh1*Δ null mutant strain (CLA6) digested with *Eco*RI-*Sal*I. (B) Lanes: 1, wild-type strain (CLA4); 2, *gdh3*Δ null mutant strain (CLA7) digested with *Hin*dIII. (C) Lanes: 1, wild-type strain (CLA4); 2, *glt1*Δ null mutant (CLA5) digested with *Afl*II. Samples in panel A were probed with a 450-bp PCR fragment internal to the *GDH1* gene, samples in panel B were probed with a 760-bp PCR fragment internal to the *GDH3* gene, and samples in panel C were probed with a 4.5-kb fragment internal to the *GLT1* gene.

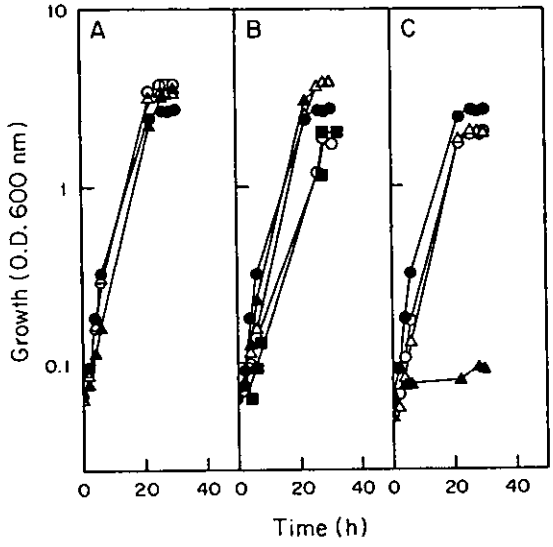

FIG. 2. (A) Growth of strains CLA4 (*GDH1 GDH3 GLT1*) (●), CLA5 (*GDH1 GDH3 glt1*Δ) (○), CLA6 (*gdh1*Δ *GDH3 GLT1*) (▲), and CLA7 (*GDH1 gdh3*Δ *GLT1*) (△). Cells were incubated on minimal medium with 40 mM $(NH_4)_2SO_4$. (B) Growth of strains CLA4 (*GDH1 GDH3 GLT1*) (●), CLA8 (*gdh1*Δ *GDH3 glt1*Δ) (○), CLA9 (*GDH1 gdh3*Δ *glt1*Δ) (▲), CLA10 (*gdh1*Δ *gdh3*Δ *GLT1*) (△), and DAN2 (*gdh1 GDH3 glt1*Δ) (■). Cells were incubated on minimal medium with 40 mM $(NH_4)_2SO_4$. Cultures of strain DAN2 were supplemented with 0.2 mM uracil. (C) Growth of strains CLA4 (*GDH1 GDH3 GLT1*) (●) and DAN3 (*gdh1 gdh3*Δ *glt1*Δ) (▲) on minimal medium with 40 mM $(NH_4)_2SO_4$ and strains CLA4 (○) and DAN3 (△) on minimal medium with 5 mM glutamate as the sole nitrogen source. O.D., optical density.

1.37-kb open reading frame showing 75.9% nucleotide sequence identity to *GDH1*; it was termed *GDH3* (3). In order to determine whether *GDH3* was responsible for the growth observed when the double mutant strains CLA8 and DAN2 were grown on ammonium, two pairs of deoxyoligonucleotides based on the nucleotide sequence of the *GDH3* open reading frame (3) were prepared: G1, 5'TTCTGTGGAGGATTCCA AAA3', and G2, 5'CGGAAGCGATATCGTGA3', from positions 29029 to 29048 and 29791 to 29775 of chromosome I, respectively. PCR amplification of yeast genomic DNA was carried out in a PTC-100 thermocycler (MJ Research, Inc.). A 760-bp *GDH3* PCR product was gel purified and cloned into the *Bam*HI-*Sal*I site of either YIp5 (24), harboring the *URA3* yeast gene, or YIp351 (9), harboring the *LEU2* yeast gene, generating plasmids pLV5 and pLV6. Both subclones were sequenced by primer extension with the Sequenase V.2 kit (USB), confirming that we had specifically amplified a *GDH3* fragment. Plasmids pLV5 and pLV6 were linearized after *Bgl*II digestion and used to direct homologous recombination into the yeast genome. This allowed the construction of *gdh3*Δ null mutant strains CLA7 (*GDH1 gdh3*Δ *GLT1*), CLA9 (*GDH1 gdh3*Δ *glt1*Δ), and CLA10 (*gdh1*Δ *gdh3*Δ *GLT1*). The triple mutant strain DAN3 (*gdh1 gdh3*Δ *glt1*Δ) was constructed from strain DAN2 by *GDH3* disruption due to pLV6 insertion; DAN2 already carried the *gdh1* EMS point mutation and the *glt1*Δ null allele (Table 1). Southern analysis was carried out with the 760-bp *GDH3* insert as a probe. The pattern of transformants clearly indicated that the insertion had occurred in the wild-type genomic sequence of *GDH3* (Fig. 1).

As can be seen in Fig. 2A and B, both the single *gdh3*Δ mutant and the double *glt1*Δ *gdh3*Δ mutant showed no growth phenotype compared to the wild-type strain. The double null mutant strain CLA10 (*gdh1*Δ *gdh3*Δ) had a 4.5-h duplication time, the same phenotype displayed by the single *gdh1* (EMS) (7) and *gdh1*Δ mutants (Fig. 2A and B). However, the triple *gdh1 gdh3*Δ *glt1*Δ (DAN3) (Fig. 2C) mutant behaved as a full glutamate auxotroph, indicating that the growth observed when the double *gdh1*Δ *glt1*Δ (CLA 8) and *gdh1 glt*Δ (DAN2) mutants were grown on ammonium was due to the presence of *GDH3*.

$NADP^+$-GDH and GOGAT activities were determined as described previously (5, 7) in extracts obtained from strains harboring various combinations of the *gdh1*, *gdh1*Δ, *gdh3*Δ, and *glt1*Δ mutations. Cells were allowed to grow to an optical density at 600 nm of 0.8 to 1.0 and collected by centrifugation. Soluble extracts were prepared by suspending whole cells in their corresponding extraction buffers (5, 7) and grinding them with glass beads in a Vortex mixer. It was found that both the EMS-derived *gdh1* strain and the *gdh1*Δ mutant showed residual $NADP^+$-GDH activity, which increased 12- to 15-fold in the absence of GOGAT (Table 2). The double mutant carrying mutations on both *GDH1* and *GDH3* completely lacked $NADP^+$-GDH activity (Table 2). Moreover, the $NADP^+$-GDH activity detected in the wild-type strain was consistently higher than that found in a *GDH1 gdh3*Δ mutant. Thus, we have concluded that the residual $NADP^+$-GDH activity observed in a GDH1-less mutant corresponds to a $NADP^+$-GDH isoenzyme encoded by *GDH3* ($NADP^+$-GDH3). The fact that the $NADP^+$-GDH activity was higher in a double mutant impaired in both *GDH1* and *GLT1* than that found in a *gdh1 GLT1* mutant (Table 2) suggests that *GDH3* expression may be down regulated by glutamate. Two observations completely negate the possibility that the $NADP^+$-GDH activity found in a *GDH1* mutant could be attributed to a leaky phenotype: first, null and EMS-derived *GDH1* mutations still show $NADP^+$-GDH residual activity; second, disruption of *GDH3* in a *gdh1* background completely abolishes $NADP^+$-GDH activity.

TABLE 2. $NADP^+$-GDH and GOGAT specific activities[a]

Strain (relevant genotype)	Sp act	
	$NADP^+$-GDH[b]	GOGAT[c]
CLA4 (*GDH1 GDH3 GLT1*)	300 (43)	26 (9)
CLA5 (*GDH1 GDH3 glt1*Δ)	420 (83)	ND[d]
CLA6 (*gdh1*Δ *GDH3 GLT1*)	1.6 (0.38)	52 (18)
CLA7 (*GDH1 gdh3*Δ *GLT1*)	190 (28)	25 (1.6)
CLA8 (*gdh1*Δ *GDH3 glt1*Δ)	24 (6)	ND
CLA9 (*GDH1 gdh3*Δ *glt1*Δ)	220 (30)	ND
CLA10 (*gdh1*Δ *gdh3*Δ *GLT1*)	ND	50 (16)
CN31 (*gdh1 GDH3 GLT1*)	2.0 (0.40)	47 (17)
DAN2 (*gdh1 GDH3 glt1*Δ)	25 (8)	ND

[a] For measurement of $NADP^+$-GDH and GOGAT, cells were grown to an optical density at 600 nm of 0.8 with 40 mM $(NH_4)_2SO_4$.
[b] Expressed as nanomoles of NADPH oxidized per minute per milligram of protein (15). The reported values are the means of five experiments. Numbers in parentheses are standard deviations.
[c] Expressed as nanomoles of NADH oxidized per minute per milligram of protein. The reported values are the means of five experiments. Numbers in parentheses are standard deviations.
[d] ND, not detected.

The fact that only triple mutants impaired in *GDH1*, *GDH3*, and *GLT1* are glutamate auxotrophs shows that *S. cerevisiae* has three pathways for glutamate biosynthesis and that all of them must be abolished in order to attain full glutamate auxotrophy.

The presence of two pathways for glutamate biosynthesis in several microorganisms has stimulated discussion on the need for two routes for the biosynthesis of the same end product. The role of these two pathways has been clearly elucidated in *Klebsiella aerogenes* and *Neurospora crassa*. In *K. aerogenes*, the GS-GOGAT pathway functions to assimilate ammonium and synthesize glutamate when the ammonium concentration is low, while GDH plays this role when the cells are cultivated under ammonium excess (17, 25). In *N. crassa*, it has been shown that the main function of GOGAT is the recycling of some organic nitrogen from glutamine to glutamate and that both GDH and GOGAT are involved in glutamate synthesis either under excess or limiting amounts of ammonium (14). For *Escherichia coli*, it has been recently proposed that glutamate biosynthesis may proceed through $NADP^+$-GDH when the cell is energy limited, while the GS-GOGAT pathway functions when the cell is not under energy limitation (8). The roles of GDH and the GS-GOGAT pathways have been studied in some yeasts. For *Candida albicans*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis*, evidence indicates that the GS-GOGAT pathway is the major pathway for ammonium assimilation (1, 11, 26). Regarding the role that *GDH1*, *GDH3*, and *GLT1* play in glutamate biosynthesis in *S. cerevisiae*, our results indicate that, in the presence of *GDH1*, the lack of either *GDH3* or *GLT1* does not result in partial glutamate auxotrophy, suggesting that *GDH1* constitutes the major pathway for glutamate biosynthesis. Simultaneous impairment of *GDH1* and *GLT1* does not result in full glutamate auxotrophy, and double mutants are able to grow on ammonium. Strains lacking *GDH1* and *GDH3* display the same phenotype as that found for single *GDH1* mutants, that is, they are both glutamate prototrophs. These results indicate that glutamate biosynthesis is mainly achieved through $NADP^+$-GDH1 and GOGAT.

The isolation of strains partially deleted in *GDH3* has been reported by Wilkinson et al. (27). Those authors concluded

that *GDH3* is not involved in glutamate biosynthesis; this finding was probably due to the fact that they used *GDH1 GLT1* strains to analyze the role of *GDH3*. By contrast, our results show that *GDH3* does play a physiologically significant role in glutamate biosynthesis, allowing considerable growth of the *gdh1 glt1* double mutant in ammonium. However, the individual contributions of $NADP^+$-GDH1, $NADP^+$-GDH3, and GOGAT in glutamate biosynthesis under different physiological conditions remain to be addressed. Finally, the use of the same cofactor and substrates by $NADP^+$-GDH1 and $NADP^+$-GDH3 and the high similarity index in their gene sequences argue in favor of divergent evolution of one from the other and perhaps of differences in expression as needed according to physiological cell conditions. We are currently purifying *GDH3* and characterizing its promoter in order to address this matter.

To our knowledge, *S. cerevisiae* is, so far, the only organism having three pathways for glutamate biosynthesis. Since a lack of either GDH or GOGAT does not result in glutamate auxotrophy in most microorganisms, selection of mutants affected in these two pathways has been done by using phenotypic traits supposedly associated with the lack of either GDH or GOGAT. This kind of selection may be biased and in some cases could have resulted, as in *S. cerevisiae*, in the isolation of regulatory mutants affecting more than one pathway for glutamate biosynthesis. Further analysis of the glutamate auxotrophs that have been obtained in other microorganisms may help uncover the existence of additional pathways for glutamate biosynthesis.

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Capítulo VI

A NADP-glutamate dehydrogenase mutant of the petit-negative yeast *Kluyveromyces lactis* uses the glutamine synthase-glutamate synthase pathway for glutamate biosynthesis.

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A NADP-glutamate dehydrogenase mutant of the petit-negative yeast *Kluyveromyces lactis* uses the glutamine synthetase-glutamate synthase pathway for glutamate biosynthesis

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The activities of the enzymes involved in ammonium assimilation and glutamate biosynthesis were determined in wild-type and NADP-glutamate dehydrogenase (GDH) null mutant strains of *Kluyveromyces lactis*. The specific NADP-GDH activity from *K. lactis* was fivefold lower than that found in *Saccharomyces cerevisiae*. The glutamine synthetase (GS) and glutamate synthase (GOGAT) activities were similar to those reported in *S. cerevisiae*. The NADP-GDH null mutant was obtained by transforming the *uraA* strain MD2/1 with a linearized integrative yeast vector harbouring a 390 bp fragment of the NADP-GDH structural gene. This mutant grew as well as the parent strain on ammonium, but showed GS and GOGAT activities higher that those found in the wild-type strain, implying that the GS-GOGAT pathway could play a leading role in glutamate biosynthesis in *K. lactis*. Southern blotting analysis of *K. lactis* chromosomes separated by contour-clamped homogeneous electric field electrophoresis, indicated that the NADP-GDH structural gene is localized on chromosome VI.

Keywords: nitrogen metabolism, glutamate biosynthesis

INTRODUCTION

Two pathways for ammonium assimilation and glutamate biosynthesis have been found in a variety of organisms. In one pathway, NADP-glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4) catalyses the reductive amination of 2-oxoglutarate to form glutamate (Holzer & Schneider, 1957). The existence of an alternative pathway for the net biosynthesis of glutamate, was demonstrated by Tempest *et al.* (1970). In this pathway, glutamate is aminated to form glutamine by glutamine synthetase (GS; EC 6.3.1.2) and the amide group is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT; EC 1.4.1.13), resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The GS-GOGAT pathway has been found in several micro-organisms (Senior, 1975; Hummelt & Mora, 1980; Bravo & Mora, 1988; Marqués *et al.*, 1992) and in higher plants (Miflin *et al.*, 1980).

The presence of two pathways for glutamate biosynthesis has opened a discussion as to the need for two routes for the biosynthesis of the same end-product. In plants, the GS-GOGAT pathway constitutes the main route of ammonium assimilation (Miflin *et al.*, 1980). In *Klebsiella aerogenes*, the GS-GOGAT pathway functions when the ammonium concentration is low, while the GDH pathway is more active when the cells are cultivated under ammonium excess (Tempest *et al.*, 1970; Meers *et al.*, 1970). In *Neurospora crassa*, it has been shown that the main function of GOGAT is to recycle some organic nitrogen from glutamine to glutamate, and that both pathways are involved in glutamate synthesis either on excess or limiting ammonium (Lomnitz *et al.*, 1987).

In *Escherichia coli*, GDH and GOGAT activities are both present, but their precise functions are not known (Senior, 1975). It had been proposed that in this bacterium, the lack of GOGAT resulted in the incapacity to use a variety of nitrogen sources (Ntr^- phenotype). However, it has now been established that the induction of Ntr enzymes is independent of GOGAT activity and mutants lacking GOGAT activity which are able to induce histidase have been isolated (Castaño *et al.*, 1992). Recently, Helling (1994) proposed that in *E. coli*, glutamate biosynthesis

Abbreviations: CHEF, contour-clamped homogeneous electric field; GS, glutamine synthetase; GOGAT, glutamate synthase.

could proceed through NADP-GDH when the cell is energy-limited, while the GS-GOGAT pathway would function when the cell is not energy-limited.

The role of the GDH and GS-GOGAT pathways has been studied in some yeasts. Activities of the two ammonium-assimilation pathways in continuous cultures of *Candida albicans* and the isolation of *Schizosaccharomyces pombe* glutamate auxotrophs has indicated that the GS-GOGAT pathway is the major pathway for ammonium assimilation in these yeasts (Holmes *et al.*, 1989; Barel & MacDonald, 1993). *Saccharomyces cerevisiae* mutants devoid of the biosynthetic NADP-GDH or of GOGAT have also been obtained (Folch *et al.*, 1989; Miller & Magasanik, 1990; Cogoni *et al.*, 1995). Those strains impaired in NADP-GDH show a twofold slower doubling time as compared to the wild-type strain when these are grown on ammonium as sole nitrogen source (Folch *et al.*, 1989). The lack of GOGAT activity shows no phenotype when this yeast is grown on either high or low ammonium (Folch *et al.*, 1989). Thus, in *S. cerevisiae* NADP-GDH has been assigned a role in glutamate biosynthesis, while the participation of GOGAT has remained unclear, and this activity would seem dispensable.

We report here catalytic activities of NADP-GDH, GS and GOGAT and the isolation of a NADP-GDH null mutant from *Kluyveromyces lactis*. The NADP-GDH-less mutant strain has no growth-defective phenotype and shows higher GOGAT and GS activities than the wild-type strain, indicating that in this yeast the GS-GOGAT pathway could play a major role in glutamate biosynthesis and ammonium assimilation.

METHODS

Growth conditions. Strains were routinely grown on minimal medium (MM) containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco). Glucose (2%, w/v) was used as the carbon source, and 38 mM $(NH_4)_2SO_4$ was used as the nitrogen source. Amino acids needed to satisfy auxotrophic requirements were added at 0·01% (w/v). Cells were incubated at 30 °C with agitation. Growth was monitored by measuring optical density at 600 nm (Hewlett Packard model 8452A spectrophotometer).

Determination of NADP-GDH, GS and GOGAT activities. Soluble extracts for enzyme assays were prepared by grinding whole cells suspended in 0·1 M potassium phosphate, pH 7·5, 1 mM EDTA, with glass beads and a Vortex mixer (six cycles of 1 min). NADP-GDH, GS and GOGAT were assayed by the methods of Doherty (1970), Ferguson & Sims (1974) and Roon *et al.* (1974), respectively.

Oligonucleotide design and PCR amplification of *K. lactis* genomic DNA. Two pairs of degenerated deoxyoligonucleotides were designed based on the amino acid sequence of *S. cerevisiae* GDH (Nagasu & Hall, 1985; Moye *et al.*, 1985) and *K. lactis* codon usage (Lloyd & Sharp, 1993).

The deoxyoligonucleotide F1, TT(G+A) GA(A+G) GA(C+T) TC(T+C) AC(T+C) CT(T+C) TT(T+C) GA was designed from the sequence LEDSTLFE located at position 17–24 in the GDH sequence. The deoxyoligonucleotide R1, TT(C+T) AA(G+A) GC(G+A) GC(G+A) TA(T+C) TG(A+G) GC(A+G) AC(G+A) TT was designed from the sequence NVAQYAALK located at position 227–235. The deoxyoligonucleotide F2, TT(G+A) GA(G+A) CA(A+G) AT(C+T) GT(C+T) AA(C+T) GA(G+A) TA(C+T) TC was designed from the sequence LEQIVNEYS located at position 277–285. The deoxyoligonucleotide R2, CAT (A+G)AT (T+C)CT (T+C)TT (C+T)AA (C+T)TC (T+C)TG (G+A)TC was designed from the sequence DQELKRIM located at position 400–407.

Total DNA from *K. lactis* strain WM37 (*MAT* a, *his3*) was used as template for amplification by PCR. It was carried out in a Coy TempCycler II with the following program: one denature cycle for 10 min at 94 °C, followed by 50 cycles of 30 s denaturation at 94 °C, 45 s annealing at 45 °C and 2 min extension at 72 °C, with a final 10 min extension at 72 °C. A 390 bp PCR product obtained from primers F2–R2 was gel-purified and ligated into the pCRII vector (Invitrogen). This subclone was sequenced by primer extension using the sequenase V.2 kit (USB).

Chromosome separation. Chromosomes from strain WM37 were separated by contour-clamped homogeneous electric field (CHEF) electrophoresis as described by Miranda *et al.* (1995).

Southern blot analysis. The agarose gels containing *Bgl*II restricted DNA from the wild-type and NADP-GDH null mutant and that containing separated chromosomes were exposed to short wavelength UV for 5 min to break the DNA, then denatured, neutralized and transferred to nylon membranes as described in Sambrook *et al.* (1989). DNA blots were probed with the 390 bp PCR fragment labelled with [α^{32}P]dCTP.

RESULTS AND DISCUSSION

Determination of the enzymes involved in glutamate biosynthesis

Glutamate biosynthesis can be achieved through the action of the NADP-GDH or through the concerted action of GS and GOGAT. To determine whether these two pathways were present in *K. lactis* the activity of these enzymes was measured (Fig. 1). In extracts of the wild-type strain grown on ammonium as sole nitrogen source, GS activity increased slightly in the stationary growth phase, while GOGAT activity was highest during the exponential growth phase. The specific activities of these two enzymes were similar to those found in *S. cerevisiae* (González *et al.*, 1985a; Folch *et al.*, 1989). NADP-GDH showed the highest activity during exponential phase of *K. lactis*, although the specific activity was fivefold lower than that reported for *S. cerevisiae* (González *et al.*, 1985b). It is worth noting that even though *K. lactis* showed a considerably lower NADP-GDH activity than *S. cerevisiae*, both strains grew on ammonium with the same doubling time (2 h). This result implies that in *K. lactis* the growth rate on ammonium is not limited by the capacity to assimilate ammonium through the NADP-GDH, but that the normal assimilation of ammonia in *K. lactis* could depend on the GS-GOGAT pathway. To resolve this question, *K. lactis* mutants devoid of NADP-GDH activity were isolated.

Isolation and characterization of a NADP-GDH null mutant

Based on the observation that there is a high degree of amino acid sequence similarity between various proteins from *S. cerevisiae* and *K. lactis* (Hendriks *et al.*, 1992) and

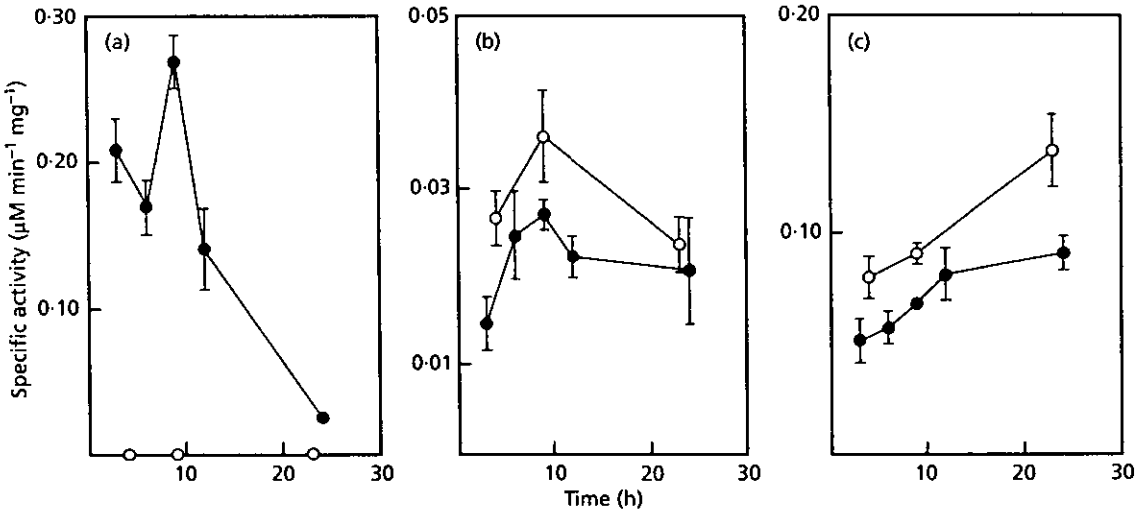

Fig. 1. Specific activities of (a) NADP-GDH, (b) GOGAT and (c) GS in the wild-type strain (●) and in the mutant strain JR1 (○). Extracts were obtained from cells grown on $(NH_4)_2SO_4$ as sole nitrogen source. Bars represent SD of four experiments.

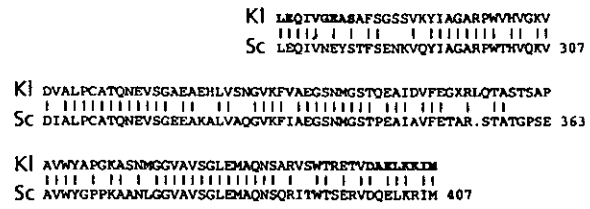

Fig. 2. Amino acid sequence alignment of *K. lactis* (Kl) PCR-amplified NADP-GDH fragment with NADP-GDH protein from *S. cerevisiae* (Sc).

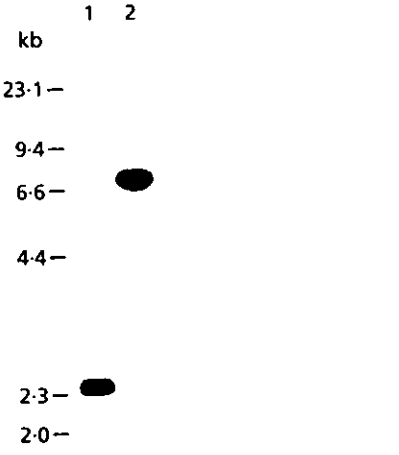

Fig. 3. Southern analysis of DNA digested with *Bgl*II obtained from *K. lactis*. Lanes: 1, wild-type strain; 2, mutant strain JR1. The blot was probed with a 390 bp PCR fragment internal to the NADP-GDH gene.

the fact that codon usage is very similar in these two yeasts (Lloyd & Sharp, 1993), two pairs of deoxyoligonucleotides were designed as amplification primers to be used on total DNA from *K. lactis* strain WM37. According to the *S. cerevisiae* NADP-GDH sequence (Moye *et al.*, 1985; Nagasu & Hall, 1985), primers F1/R1 (see Methods), were expected to amplify a 654 bp fragment; primers F2/R2 were expected to amplify a 390 bp fragment, while the combined primers F1–R2 were expected to amplify a 1170 bp fragment. Only the F2/R2 couple amplified the expected product. This 390 bp fragment was subcloned into the pCRII vector and sequenced in its entirety. The deduced amino acid sequence showed a high degree of similarity (75% similarity, 67% identity) with the corresponding region of the NADP-GDH protein from *S. cerevisiae* (Fig. 2).

To obtain a *K. lactis* NADP-GDH null mutant, we constructed plasmid pJR1 by cloning the 390 bp PCR fragment into YIp352 integrative vector, which harbours the *URA3* yeast marker. The resulting plasmid, pJR1, was linearized by digesting at the single *Cla*I site located at position 214 of the PCR fragment. Strain MD2/1 (*MATα lysA argA uraA*) was transformed to uracil prototrophy. Chromosomal DNA was isolated from 10 transformants and digested with *Hin*dIII. Southern analysis was carried out with the 390 bp PCR fragment as the probe. The pattern of transformants clearly indicated the insertion of the construct in the wild-type genomic sequence of NADP-GDH (Fig. 3). The null mutants obtained were completely devoid of NADP-GDH activity (Fig. 1a).

The doubling time of the wild-type and of the NADP-GDH mutant strains when both were grown on ammonium as sole nitrogen source, was 120 min (data not shown). These results imply, that in *K. lactis*, NADP-GDH could be a non-essential enzyme under these growth

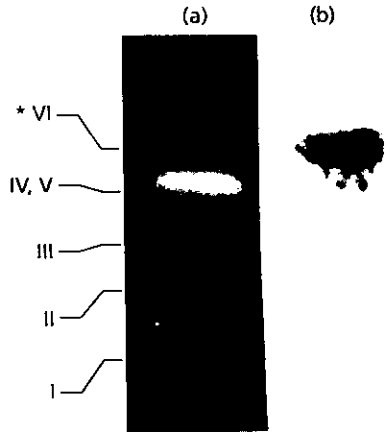

Fig. 4. (a) Chromosome pattern of the *K. lactis* strain WM37 separated by CHEF electrophoresis and stained with ethidium bromide. (b) Autoradiography of a blot showing hybridization of chromosome VI (*) to the ^{32}P-labelled 390 bp GDH PCR fragment.

conditions. GOGAT and GS specific activities were 1·5- to 2-fold higher in the NADP-GDH mutant strain than those found in the wild-type strain (Fig. 1b, c). These results imply that the GS-GOGAT pathway could be either a compensatory mechanism for the lack of NADP-GDH or the primary route of ammonium assimilation and glutamate biosynthesis and that the NADP-GDH could have only a collateral significance. In *C. albicans* and *Sc. pombe*, the GS-GOGAT pathway plays an essential role in ammonium assimilation (Holmes *et al.*, 1989; Barel & MacDonald, 1993). In *Sc. pombe*, mutants devoid of GOGAT activity grow slowly and increase fivefold their NADP-GDH levels implying that the GS-GOGAT pathway is the primary route of glutamate biosynthesis (Barel & MacDonald, 1993). It can be concluded that while the yeasts studied to date have both routes for glutamate biosynthesis, the primary route is not always the same.

Chromosomal localization of the NADP-GDH structural gene

Using the CHEF conditions described in Methods, six bands were separated that corresponded to chromosomes from strain WM37 (*MAT*a, *his3*) (Fig. 4, a), that match with those reported from the strain CBS 2360 (Sor & Fukuhara, 1989). These chromosomes, numbered I to VI, were immobilized on a nylon membrane. The *K. lactis GDH1* (NADP-GDH) gene was localized on chromosome VI using the PCR-amplified fragment as probe (Fig. 4, b). The *K. lactis GDH1* gene encoding the NADP-GDH should be added to the growing list of genes mapped in this organism.

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Capítulo VII

RESULTADOS Y DISCUSION

Caracterización bioquímica de la enzima GOGAT de *S. cerevisae*, análisis de la secuencia de aminoácidos de esta proteína, y regulación transcripcional del gen *GLT1*.

La purificación de la enzima GOGAT de *S. cerevisiae* mostró que es un homotrímero formado por subunidades de 200 kD. La masa molecular del monómero es similar a la reportada para las enzimas GOGAT de organismos eucariontes, y diferente a la reportada para las enzimas GOGAT de eubacterias, de las que se han purificado enzimas NADPH-GOGAT formadas por dos subunidades diferentes, y una enzima NADH-GOGAT formada por cinco subunidades diferentes (para una revisión ver Valenzuela, 1994). Recientemente se purificó la proteína GOGAT de la arqueobacteria *Pyrococcus* sp., la cual está formada por un homotetrámero constituido por subunidades de 53 kD, similares a la subunidad pequeña de la GOGAT de *E. coli* (Jongsareejit y cols., 1997).

La clonación del gen *GLT1* y la mutagénesis dirigida del mismo, permitió obtener mutantes de *S. cerevisiae* carentes de actividad de GOGAT, que afectan al gen estructural. El análisis de estas cepas permitió, por un lado, comprobar por métodos genéticos la existencia de una sola enzima con actividad de GOGAT en *S. cerevisiae*, y por otro, demostrar que el gen *GLT1* codifica para esta proteína.

La secuencia de nucleótidos del gen *GLT1* codifica para una proteína de 2141 aminoácidos y no contiene intrones. La comparación de la secuencia de aminoácidos de

la enzima GOGAT de *S. cerevisiae* con las secuencias reportadas para las enzimas GOGAT de *E. coli* (Gosset y cols., 1989), *Medicago sativa* (Gregerson y cols., 1993) y *Zea mais* (Sakakibara y cols., 1991), mostró que existe una alta conservación entre ellas, identificándose los dominios probables de unión a glutamina, a los cofactores FMN y NADH, y al grupo fierro azufre (3Fe-4S) en la enzima GOGAT de *S. cerevisiae*. El dominio de unión a glutamina de GOGAT, con un residuo de cisteína en la posición uno, es homólogo al de enzimas amido-transferasas de la familia Ntn-hidrolasas (Kim y cols., 1996). Al analizar la secuencia amino-terminal de la proteína y la secuencia de aminoácidos deducida a partir de la secuencia de nucleótidos de *GLT1*, se encontró que esta enzima, al igual que todas las enzimas GOGAT descritas (Sakakibara y cols., 1991; Pelanda y cols., 1993; Gregerson y cols., 1993), contiene una presecuencia, cuya longitud es de 53 aminoácidos; ésta es una característica que comparten las enzimas GOGAT con enzimas PRPP amidotransferasas (Kim y cols., 1996). Gosset y cols. (1989), proponen que posiblemente un proceso proteolítico similar al que ocurre en la PRPP de *B. subtilis*, es el responsable de generar el amino-terminal maduro de la subunidad grande de la enzima GOGAT de *E. coli*. Un proceso similar podría ocurrir en la maduración de la enzima GOGAT de *S. cerevisiae*.

La presecuencia de la enzima Fd-GOGAT de plantas posiblemente funciona como señal que localiza a la proteína en los cloroplastos. Por otro lado, la mayor parte de la actividad de NADH-GOGAT de nódulos de raíz se ha encontrado también en plastos; sin embargo, existe mayor similitud de la presecuencia de esta proteína a una señal de localización en mitocondrias. No se ha determinado si en realidad esta secuencia corresponde a una señal de localización (Vance y cols., 1995). La presecuencia de la enzima GOGAT de *S.*

cerevisiae no presenta características comunes con las señales de importación a mitocondria.

Los resultados obtenidos en cepas silvestres de *S. cerevisiae* que crecen en etanol como única fuente de carbono, mostraron que existe mucho mayor actividad de beta-galactosidasa producida por una fusión *GLT1-lacZ*, respecto a la actividad de la enzima GOGAT presente en la célula (datos no mostrados). Estos resultados sugieren que existe control post-transcripcional de la enzima GOGAT. Una posibilidad donde se puede llevar a cabo este control es durante el proceso de maduración de la enzima; la presecuencia permitiría mantener un reservorio de enzima inactiva en la célula.

El gen que codifica para la enzima NADH-GOGAT de alfalfa comprende 22 exones interrumpidos por 21 intrones. La presecuencia se localiza en el primer exón, la región que corresponde a la subunidad grande de *E. coli* está codificada por los exones 1 a 16, y la región que corresponde a la subunidad pequeña de *E. coli* está codificada por los exones 17 a 22. La región hidrofílica que une a estas dos regiones está codificada por los exones 16 y 17, los cuales están separados por un intrón (Vance y cols., 1995). La organización del gen que codifica para los dominios funcionales de la proteína NADH-GOGAT de alfalfa y de la enzima NADH-GOGAT de *S. cerevisiae*, es similar a la organización del operón *gltBD* de *E. coli* (Gosset y cols., 1989), del operón *gltBD* de *K. aerogenes* (Macaluso y cols., 1990), y del operón *gltAB* de *B. subtilis* (Bohannon y Sonenshein, 1989). En contraste a esta organización, el operón *gltDB* de *Azospirillum brasilense* contiene en primer lugar al gen que codifica para la subunidad pequeña (Pelanda y cols., 1993). Tomando en cuenta por un lado que en procariontes la enzima GOGAT está formada por subunidades diferentes, y por otro, que la organización de

algunos operones bacterianos es similar a la organización del gen *GLT1* y al de la enzima GOGAT de alfalfa, se puede sugerir que el gen eucarionte podría ser el producto de una fusión génica.

Al clonar el gen *GLT1* se obtuvo su región promotora, la cual incluye aproximadamente 700 pb río arriba del inicio de traducción de *GLT1*. Esta región se fusionó al gen reportero *lacZ* para estudiar el control transcripcional de *GLT1*. Los resultados mostraron que la expresión del gen *GLT1* está controlada negativamente por glutamato, el producto de la reacción de GOGAT. Se ha reportado que este compuesto reprime la transcripción de los genes que codifican para la enzima GOGAT en las bacterias *E. coli* (Castaño y cols., 1992) y *B. subtilis* (Bohannon y cols., 1985). En condiciones de exceso de glutamato, la expresión de *GLT1* está gobernada tanto por represión mediada por glutamato, como por activación llevada a cabo por Gln3p y Gcn4p; como resultado se obtiene una transcripción disminuida pero no nula de la expresión de *GLT1*. Cuando existe limitación de glutamato la expresión de *GLT1* se regula por Gln3p, Gcn4p y posiblemente por un factor de la familia Cys_6-Zn_2. En estas condiciones, ocurre aumento de la transcripción por disminución de la represión y la acción de los activadores.

El regulador Gcn4p participa en la expresión de *GLT1* cuando se crecen a las células en medio mínimo (expresión basal), y en la expresión activada en respuesta a condiciones de limitación de aminoácidos; por lo que el gen *GLT1* se encuentra regulado, junto con otros genes que codifican para enzimas biosintéticas de aminoácidos, por el control general de aminoácidos (GCN). En condiciones de limitación de aminoácidos, los genes regulados por el sistema GCN aumentan su expresión de 2 a 10 veces (Hinnebusch, 1986). En ausencia de Gcn4p, la transcripción de *GLT1* disminuye a la mitad en condiciones

basales, y cuatro veces en condiciones de limitación de aminoácidos (ver la Tabla 3 del capítulo IV), por lo que este regulador participa moderadamente en la regulación de *GLT1*.

Cuando está presente una fuente primaria de nitrógeno, la actividad de Gln3p se previene por Ure2p o por otra proteína (Coschigano y Magasanik, 1991). Sin embargo, la expresión de *GLT1* en glutamina o asparagina depende del regulador Gln3p. Una explicación a estos resultados es que Gln3p posiblemente es capaz de activar la expresión de *GLT1* aún en presencia de represión nitrogenada, al actuar cooperativamente con un regulador específico, como se ha descrito en la regulación del promotor de *GLN1* y *PUT1* (Rai y col., 1995). El activador específico podría ser una proteína que reconozca a la secuencia palindrómica 5'-CGG-N_{15}-CCG-3' localizada cerca del elemento GATA1 (ver la Fig. 1 del capítulo IV). De acuerdo con esta hipótesis, los resultados obtenidos al ir eliminando regiones en el extremo 5' del promotor sugieren una función activadora para la secuencia palindrómica (ver la Fig. 3 del capítulo IV). Para definir si realmente esta secuencia está involucrada en la regulación de *GLT1*, y si se encuentra actuando junto con la secuencia GATA1, que es reconocida por el factor Gln3p, se puede efectuar la mutagénesis dirigida de las secuencias CGG y GATA1, y medir los niveles de expresión del gen reportero. El factor que une a la secuencia palindrómica posiblemente constituye un control específico de regulación del gen *GLT1*, por lo que su caracterización es importante. Para clonar el gen que codifica para este regulador, se puede realizar el escrutinio de una biblioteca de expresión de proteínas de *S. cerevisiae*, con una sonda marcada que contenga la secuencia palindrómica.

La represión del gen *GLT1* posiblemente está mediada por proteínas reguladoras específicas que se unen a las regiones URR1 y URR2. Sin embargo, la maquinaria transcripcional unida al sitio de inicio de transcripción y a la caja TATA del gen *UGA3* -adyacente a *GLT1*-, o algún regulador de la expresión de UGA3, podrían ser los responsables de la represión mediada por la región URR1. A este respecto, es importante saber cuáles son las regiones donde se estructura el complejo de inicio de transcripción del gen *UGA3*, qué elementos en *cis* y factores en *trans* regulan la expresión de este gen, y si los genes *GLT1* y *UGA3* se encuentran corregulados o no. Por otro lado, realizar un análisis por mutagénesis dirigida y ensayos de retardo en gel pueden ayudar a definir si se une una proteína represora a la región URR1. La región represora URR2 es muy amplia, por lo que para determinar la o las regiones involucradas en la represión, se pueden eliminar secuencias internas en la región URR2 y medir la expresión del gen reportero. Con el fin de identificar el gen que codifica para el represor putativo de *GLT1* se podrían buscar mutantes constitutivas en *trans*, que en presencia de glutamato presenten altos niveles de expresión de *GLT1* o de un gen reportero.

Respecto al mecanismo de represión, existen varias preguntas: ¿cómo se transmite la señal de exceso de glutamato al mecanismo represor de la transcripción de *GLT1*?, ¿cuál es el mecanismo de represión: están involucradas la estructura de la cromatina y/o desacetilación de histonas, o existe competencia entre activadores y represores en la unión al DNA y/o a la maquinaria transcripcional? Los resultados de regulación de *GLT1* por el complejo ADA, obtenidos recientemente en nuestro laboratorio por Simón Guzmán (1998), sugieren que la activación de la transcripción de *GLT1* involucra la acetilación de la cromatina. Sería interesante definir la o las proteínas activadoras con la

cuales el coactivador ADA interacciona para ayudar a formar el complejo de inicio de transcripción, e intervenir en la reorganización nucleosomal de la región promotora de *GLT1*. Posiblemente una de estas proteínas es Gcn4p, la cual requiere de la función del complejo ADA para promover los niveles máximos de expresión de algunos genes biosintéticos de aminoácidos (Georgakopoulos y Thireos, 1992).

Los elementos poli(dA-dT) pueden facilitar la unión de los activadores al DNA (Iyer y Struhl, 1995b); o bien, unir a un regulador positivo específico (Lue y cols., 1989). En el promotor de *GLT1* existen regiones poli(dA-dT) imperfectas cercanas a sitios débiles de unión a Gcn4p y a los sitios de inicio de transcripción. Cambiar la distancia entre estas secuencias podría definir si funcionan permitiendo el acceso de Gcn4p o TBP al DNA nucleosomado. Otra posibilidad es que su acción sea mediada no por el efecto de su estructura sobre el nucleosoma, sino por la unión de proteínas a esta secuencia. Esta unión se podría demostrar por ensayos de retardo en gel y por mutagénesis dirigida de las regiones poli(dA-dT).

Algunos promotores de levadura contienen varias cajas TATA y diferentes inicios de transcripción. El complejo transcripcional se ensambla en uno u otro inicio dependiendo de la capacidad de las cajas TATA presentes para interaccionar con TBP, de la cercanía de las cajas TATA a los elementos *UAS*, y de la presencia, localización o calidad de elementos INR. El análisis de promotores que contienen dos cajas TATA indica que existe un uso diferencial cuando la caja TATA que se encuentra río arriba, es más débil que la caja que se encuentra río abajo. En la situación contraria, la caja TATA localizada río arriba, más fuerte, se utiliza preferentemente (Iyer y Struhl, 1995). En el promotor de *GLT1* existen dos cajas TATA y dos sitios de inicio de transcripción en amonio como

fuente de nitrógeno. El sitio de inicio de la transcripción +52 se utiliza muy poco respecto al sitio de inicio +1. Posiblemente la caja TATA1 es más fuerte que la caja TATA2, y según la hipótesis de Iyer y Struhl (1995a), existiría preferencia por ocupar la caja TATA1 y el sitio +1 para el ensamble del complejo transcripcional. Sin embargo, Harbury y Struhl (1989) demostraron que una secuencia idéntica a la caja TATA2 del promotor de *GLT1*, es capaz de funcionar como una caja TATA fuerte, abriendo la posibilidad de que exista un uso diferencial de las cajas TATA de *GLT1*.

Los fragmentos más cortos del promotor que no contienen a la caja TATA1, o que contienen a la caja TATA1 pero no a los *UAS* que se encuentran río arriba de esta caja, promueven la síntesis de RNAm a partir del inicio +52, utilizando posiblemente la caja TATA2 (ver las Figuras 1 a 3 del capítulo IV). Estos fragmentos del promotor presentan un sitio de unión a Gcn4p y regiones poli(dA-dT). Sería interesante estudiar si los sitios de inicio de transcripción +1 y +52, junto con sus respectivas cajas TATA y *UAS*, dirigen la transcripción bajo diferentes condiciones fisiológicas. Una condición en la cual se puede determinar si aumenta la síntesis de RNAm a partir del segundo inicio de transcripción (+52), es en limitación de aminoácidos.

Papel fisiológico de GOGAT en *S. cerevisiae*.

El hábitat natural de *S. cerevisiae*, provee principalmente de fuentes secundarias de nitrógeno a la levadura. En la uva, la prolina es el aminoácido predominante con una concentración de 10 a 100 veces mayor que la de otros aminoácidos. Otra fuente de nitrógeno abundante es la arginina, cuyo catabolismo requiere la conversión a prolina. Cuando el amonio, arginina y otras fuentes de nitrógeno presentes en el jugo de uva se

acaban, las levaduras utilizan la prolina remanente como única fuente de nitrógeno, bajo condiciones aeróbias de crecimiento (Gimeno y cols., 1992).

En el laboratorio, el medio mínimo con prolina como fuente de nitrógeno mimetiza el hábitat natural de la levadura (Gimeno y cols., 1992). Precisamente en estas condiciones la actividad de $NADP^+$-GDH1 disminuye (datos no mostrados) y la actividad de GOGAT aumenta (ver la Tabla 2 del capítulo IV). Nuestros resultados sobre la represión transcripcional de *GLT1* por glutamato y la activación mediada por el sistema GCN, sugieren una función de GOGAT en condiciones de limitación de glutamato, y en general cuando hay desbalance en la poza de aminoácidos. Por otro lado, los resultados sobre regulación transcripcional de *GLT1* mediada por Gln3p, muestran que este regulador participa en el aumento de los niveles de RNAm de *GLT1* en prolina como fuente de nitrógeno. El crecimiento en prolina como única fuente de nitrógeno, es una condición de desrepresión catabólica nitrogenada -donde Gln3p está activo-, y baja concentración de glutamato –donde el sistema GCN está activo-; por lo que estos datos sugieren que el ciclo GS-GOGAT puede tener una función importante en la reasimilación del amonio que proviene de la degradación de la prolina y otras fuentes secundarias de nitrógeno.

En presencia de estrés osmótico, ocurre un aumento en la concentración intracelular de glutamato como mecanismo de protección. Este aumento puede ser debido a la síntesis *de novo*, a la presencia de un almacén intracelular de este aminoácido, o bien, a la biosíntesis a partir del flujo catabólico de aminoácidos (Saroja, 1996). El papel que tienen las vías de biosíntesis *de novo* de glutamato en estas condiciones se ha estudiado muy poco; al realizar estos estudios hay que considerar la existencia de un mecanismo de activación que compita o elimine la represión mediada por el glutamato (Saroja, 1996). A este

respecto, nuestros resultados sobre el control de la transcripción de *GLT1* demostraron que se encuentra regulada por Gln3p, un regulador que está activo en glutamato (Coffman y cols., 1997) y que permite la expresión de *GLT1* aún en condiciones de represión mediada por este aminoácido. Estas características del control de la expresión de *GLT1* lo hacen un gen interesante para estudiar desde el punto de vista del estrés osmótico.

Por otro lado, la expresión baja de *GLT1* en presencia de glutamato, permite sugerir que la célula requiere una síntesis constante de este aminoácido a través del funcionamiento del ciclo GS-GOGAT. En estas condiciones, GOGAT además de sintetizar glutamato, podría estar reciclando glutamina, para mantener el funcionamiento del ciclo GS-GOGAT, con el consecuente recambio de ATP y NADH por ADP^+ y NAD^+. A este respecto se ha demostrado que mantener una relación ATP/ADP^+ baja, optimiza el catabolismo de glucosa de la célula; estos resultados permitieron sugerir que el reciclamiento de glutamina establece una relación entre el metabolismo de nitrógeno y el de carbono (Flores-Samaniego y cols., 1993). La actividad de GOGAT también podría evitar la acumulación de glutamina, un compuesto que se mantiene en baja concentración en la célula de levadura, y cuya concentración se ha demostrado que es una señal directa del estado metabólico nitrogenado en bacterias (ver la revisión de Merrick y Edwards, 1995), y posiblemente también en levaduras (ver la revisión de Magazanik, 1992).

Análisis de mutantes en las distintas vías de asimilación de amonio y biosíntesis de glutamato en *S. cerevisiae*.

El fenotipo silvestre de una cepa sin actividad de GOGAT (*glt1*), y la auxotrofía parcial por glutamato que presenta una cepa sin actividad de $NADP^+$-GDH1 (*gdh1*), en amonio como fuente de nitrógeno, permiten concluir que la vía principal de asimilación de amonio y biosíntesis de glutamato es la enzima $NADP^+$-GDH1. Al analizar dobles mutantes *glt1 gdh1*, se encontró que estas cepas aún pueden crecer en amonio como fuente de nitrógeno. Este fenotipo permitió encontrar el gen *GDH3*; el cual codifica para una isoenzima de $NADP^+$-GDH1, y que funciona como una ruta alternativa de asimilación de amonio y biosíntesis de glutamato en ausencia de las enzimas $NADP^+$-GDH1 y GOGAT. Mutantes triples *glt1 gdh1 gdh3* son totalmente auxótrofas de glutamato. El fenotipo de una cepa que está afectada en la actividad de $NADP^+$-GDH3 es similar al de una cepa silvestre, cuando se crece en amonio como fuente de nitrógeno y glucosa como fuente de carbono.

Wolfe y Shields (1997) proponen que la duplicación del genoma de *S. cerevisiae* pudo ser muy importante durante la evolución adaptativa al crecimiento anaerobio. Avendaño (1998) discute el origen de los genes *GDH1* y *GDH3* como producto de una duplicación génica, donde el promotor de cada gen diverge y permite que las enzimas se expresen en condiciones diferentes de crecimiento; la presencia de los dos genes *GDH1* y *GDH3* posiblemente confiere una ventaja adaptativa a las células. Avendaño (1998) también analiza la redundancia génica que existe para llevar a cabo la asimilación de amonio y la biosíntesis de glutamato en *S. cerevisiae*, sugiriendo que la presencia de *GLT1*, *GDH1* y *GDH3* están de alguna manera eficientizando este proceso. Esta mayor eficiencia

metabólica aumentaría la fidelidad del proceso cuando los genes trabajan juntos en alguna condición de emergencia (Thomas, 1993). Para comprender mejor la función de cada una de las vías de asimilación de amonio y biosíntesis de glutamato, y probar las hipótesis de Thomas (1993) y Avendaño (1998), se debe realizar un estudio de la regulación transcripcional de *GLT1*, *GDH1* y *GDH3* en diferentes condiciones fisiológicas: en distintas fuentes de nitrógeno y de carbono, en condiciones de aerobiosis y anaerobiosis, en condiciones de estrés. También es importante el estudio comparativo de las características bioquímicas de las enzimas $NADP^+$-GDH1 y $NADP^+$-GDH3. Algunas condiciones de estrés a las que se enfrentan las levaduras y donde sería interesante estudiar la función de las vías de asimilación de amonio y biosíntesis de glutamato, se mencionan a continuación.

Las células diploides de levadura presentan un crecimiento pseudohifal cuando crecen en medio sólido, que se induce por limitación de la fuente de nitrógeno –baja concentración de amonio, o prolina como única fuente de nitrógeno-. La colonia de levaduras forma estructuras macroscópicas que emanan hacia afuera de la colonia, posiblemente buscando nutrientes y sustrato alejados de sus sitio inicial de colonización (Gimeno y cols., 1992). Trabajando en estas condiciones, podría investigarse si la falta de GOGAT, $NADP^+$-GDH1 o $NADP^+$-GDH3 afecta el crecimiento pseudohifal del diploide.

Cuando la fuente de nitrógeno y la de carbono se terminan, la célula diploide tiene como alternativa la formación de esporas. El análisis de la capacidad de esporular de cepas diploides mutantes en *GLT1*, *GDH1* o *GDH3*, permitiría saber si alguna de estas vías tiene una función principal durante la esporulación, cuando las condiciones son de limitación de nitrógeno y de energía.

Por otro lado, las células haploides que se enfrentan a una diminución o desbalance en la poza de aminoácidos, o bien que crecen en prolina como fuente de nitrógeno, aumentan la expresión de *GLT1*, sugiriendo un papel importante de GOGAT en estas condiciones. Sin embargo, este aumento es moderado por lo que el estudio de la expresión de *GLT1*, *GDH1* y *GDH3* podría definir cual de las tres vías provee de glutamato a la célula en estas condiciones. Un estudio adicional podría incluir el crecimiento de células haploides *glt1*, *gdh1* o *gdh3*, en condiciones de limitación de nitrógeno y carbono.

Vías de asimilación de amonio y biosíntesis de glutamato en *K. lactis*.

Mediante la determinación de actividades enzimáticas se demostró la presencia de la vía NADP-GDH1 y la vía GS-GOGAT en la levadura *K. lactis*. Las cepas que carecen de actividad de $NADP^+$-GDH1 (*gdh1*) presentan un fenotipo silvestre en amonio como fuente de nitrógeno y aumentan la actividad de GOGAT y GS, por lo que posiblemente en *K. lactis*, la vía principal de asimilación de amonio y síntesis de glutamato es el ciclo GS-GOGAT. El análisis de cepas que no presenten actividad de GOGAT, puede corroborar si el ciclo GS-GOGAT es la ruta principal de asimilación de amonio y biosíntesis de glutamato, en amonio como fuente de nitrógeno.

Las cepas con interrupción en el gen *GDH1* no presentan ninguna actividad residual de NADP+-GDH1, lo que sugiere fuertemente que en la levadura *K. lactis* existe una sola enzima con actividad de $NADP^+$-GDH1. Al interrumpir los genes que codifican para las enzimas $NADP^+$-GDH1 y GOGAT en esta levadura, posiblemente se obtengan auxótrofos totales de glutamato.

En condiciones aerobias, el metabolismo de la glucosa en *K. lactis* es en su mayor parte oxidativo, mientras que el de *S. cerevisiae* es principalmente fermentativo (Wésolowski-Louvel y cols., 1996). Nuestros resultados muestran que en estas condiciones de crecimiento, la levadura *K. lactis* utiliza principalmente el ciclo GS-GOGAT para asimilar amonio y biosintetizar glutamato, mientras que *S. cerevisiae* utiliza principalmente la vía $NADP^+$-GDH1. Estos experimentos permiten iniciar un estudio comparativo de las vías de asimilación de amonio y biosíntesis de glutamato en *K. lactis* y *S. cerevisiae*.

CAPITULO VIII

CONCLUSIONES Y PERSPECTIVAS

La razón por la cual los organismos requieren diferentes rutas metabólicas para asimilar amonio y biosintetizar glutamato, es un tema que se ha estudiado en bacterias, hongos y plantas superiores. En *S. cerevisiae*, un organismo unicelular que puede crecer en amonio como única fuente de nitrógeno, se han reportado las dos vías descritas para la asimilación de amonio y para la biosíntesis de glutamato: la enzima $NADP^+$-GDH1 y el ciclo GS-GOGAT. Estudiar este organismo nos permitió realizar un análisis fisiológico de la enzima GOGAT desde el punto de vista bioquímico, genético y molecular.

Nuestro trabajo permite concluir lo siguiente: 1) la levadura *S. cerevisiae* tiene una sola enzima de 200 kD con actividad de GOGAT, la cual está codificada por el gen *GLT1*, 2) los dominios funcionales y organización estructural de esta enzima se encuentran altamente conservados, mostrando mayor similitud con la enzima de eucariontes superiores, 3) la expresión de GOGAT se regula a nivel transcripcional: la expresión de *GLT1* se reprime por glutamato, se activa por factores que pertenecen al sistema de represión catabólica nitrogenada (NCR), y por el control general de nitrógeno (GCN), 4) los resultados sobre la regulación de *GLT1* sugieren un papel importante de GOGAT en la reasimilación del amonio que proviene de la degradación de fuentes secundarias de nitrógeno, en condiciones de limitación de glutamato o de desbalance en la poza de aminoácidos, en presencia de estrés osmótico u otras condiciones donde se requiera la biosíntesis *de novo* y la acumulación de glutamato, 5) existen tres rutas de asimilación de

amonio y de biosíntesis de glutamato en *S. cerevisiae*, $NADP^+$-GDH1, $NADP^+$-GDH3 y GS-GOGAT; una cepa que presenta mutaciones en los genes *GDH1*, *GDH3* y *GLT1* es auxótrofa de glutamato, 6) cuando el amonio es la única fuente de nitrógeno, *S. cerevisiae* utiliza principalmente a la enzima $NADP^+$-GDH1 para asimilar amonio y sintetizar glutamato, 7) posiblemente en *K. lactis* la vía principal de asimilación de amonio y biosíntesis de glutamato, en amonio como fuente de nitrógeno, es el ciclo GS-GOGAT.

De estos estudios han surgido nuevas preguntas:¿porqué *S. cerevisiae* presenta tres vías de asimilación de amonio y biosíntesis de glutamato?, ¿existen solo dos rutas metabólicas involucradas en la asimilación del amonio y la biosíntesis de glutamato en *K. lactis*? ¿cuál es el papel fisiológico de la enzima $NADP^+$-GDH3?, ¿la presencia de la enzima $NADP^+$-GDH3, confiere alguna ventaja adaptativa para que *S. cerevisiae* pueda crecer en anaerobiosis?, ¿la enzima NADP+-GDH3 tiene alguna función regulatoria?, ¿existen diferencias cinéticas entre las enzimas $NADP^+$-GDH1 y $NADP^+$-GDH3?.

Queda por comprobar el posible papel fisiológico de GOGAT que se propone en esta tesis, y se sugiere estudiar el control a nivel post-transcripcional de esta enzima. Para completar los estudios sobre la regulación transcripcional del gen *GLT1*, sería importante conocer los elementos en *cis* y los factores en *trans* que permiten una regulación específica, tanto positiva como negativa, de este gen; así como estudiar el mecanismo por el cual el glutamato ejerce su efecto represor.

Continuar el estudio sobre la asimilación de amonio y biosíntesis de glutamato en la levadura *K. lactis*, permitirá comparar la fisiología de esta levadura con la de *S. cerevisiae*.

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