The invention relates to a plasmid carrying a selenium-specifying DNA sequence of the E. coli sisF gene upstream the E. coli lac\textsuperscript{+}Z gene which permits the incorporation of selenocystein into \textbeta-\textgamma-\textalpha-programmed activity. Particular plasmids according to the invention are pRM2, deposited at the ATCC under No. 75594 and pRM4, deposited at the ATCC under No. 75595. The invention also relates to micro-organisms transformed with a plasmid according to the invention having selenium-dependent \textbeta-\textgamma-\textalpha-programmed activity. The invention provides a method for the quantitative determination of selenium in selenium derivatives in a biological sample comprising incubating micro-organisms according to the invention in a suitable medium also containing said sample and measuring the level of \textbeta-\textgamma-\textalpha-programmed activity. The biological sample may be, e.g. a blood sample or a food sample.
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BIOASSAY OF SELENIUM

FIELD OF THE INVENTION
The invention relates to a bio-assay of selenium in selenium derivatives, transformed micro-organisms therefor, and plasmids suitable for generating said transformed micro-organisms.

BACKGROUND OF THE INVENTION

Furthermore, as recently discovered, selenium is also involved in the form of selenocysteine in two additional human selenium-containing proteins. The first is the thyroid enzyme type 1 iodothyronine 5' deiodinase (5' deiodinase) [Berry, M.J. et al. Nature 349, 438-440] that converts thyroxine to the active thyroid hormone. The second is the
plasma protein selenoprotein P [Read, R. et al. (1990) J.
Biol. Chem. 265, 17899-17905; Hill, K.E. et al., J. Biol.
Chem. 266, 10050-10053] which actually contains 10 seleno-
cysteine residues per subunit. Thus, selenium seems to have
biochemical functions in addition to those associated with
antioxidation, and may have a more general role in the
maintenance of human health. So far, low levels of selenium
intake have clearly been shown to be associated with only
two diseases, Keshan Disease [Yang, G. et al. (1984) in
Advances in Nutritional Research, ed. Draper, H.H. (Plenum
Press, New York) pp.203-231] and Kaschin Beck disease [Mo,
D-X. (1987) in Selenium in Biology and Medicine, eds. Combs,
G.F. et al. (Avi, Van Nostrand Reinhold, New York) pp. 924-
933], both occurring in China. However, there are claims for
the relationship between pronounced or even marginal sele-
nium deficiencies and other diseases as well [Neve, J.

In recent years there has been great interest in the field
of selenium biochemistry and genetics. In-frame TCA codons
have been found within the coding sequences of each of the
genes encoding selenoproteins including the E. coli enzyme
formate dehydrogenase (FDH) [Zinoni, F. et al. (1986) Proc.
Natl. Acad. Sci. USA 84, 3156-3160], mammalian proteins GPX
[Chambers, I. et al. (1986) EMBO J. 5, 1221- 1227; Mullen-
bach, G.T. et al. (1988) Protein Engineering 2, 239-246],
5' deiodinase [Berry et al. (1991) ibid.; Berry, M.J. et al.
(1991) Nature 353, 273-276], and selenoprotein P [Read et
al. (1990) ibid.; Hill et al. (1991) ibid.]. It is now known
that selenocysteine is coded by a UGA codon that usually
acts as a termination codon. As has been shown in E. coli,
several specific genes are involved in this incorporation
process [for review see Bock, A. et al. (1991) TIBS. 16,
463-467]. Of particular importance are the gene selB, which codes for SelB protein, and selC, which codes for tRNA^Sec. This tRNA is charged with selenocystein and is thus responsible for selenocysteine incorporation into polypeptides. In addition, a selenium-specific 47 nucleotide long codon context of the UGA codon in the E. coli FDH gene (fdhF) has been identified [Zinoni, F. et al. (1990) Proc. Natl. Acad. Sci. USA 87, 4660-4664] that permits the UGA codon to be read as a selenocysteine codon rather than as a termination codon. This context can form a stem-loop structure (as may be seen in Fig. 1E herein). Because of the presumed interaction of this stem-loop structure with SelB protein, the codon context seems to be required for selenocysteine incorporation [Heider, J. et al. (1992) EMBO J. 11, 3759-3766].

The assays currently available for determining selenium concentrations in various compounds are mainly physical or chemical methods by which the element selenium is determined directly [Ihnat, M. et al. (1986) Acta Pharmacol Toxicol. 59, 566-572; Neve, J. (1991) J. Trace Elem Electrolytes Health Dis. 5, 1-17]. However, the known prior art methods for quantitative determination of selenium require sophisticated and expensive equipment, are not very specific and are also not sensitive to very low concentrations of selenium.

The present invention offers a simple, relatively inexpensive bio-assay that relates linearly and specifically to very low concentrations of selenium in several simple selenium derivatives. The bio-assay of the invention is carried out in E. coli and employs specific novel recombinant DNA plasmid constructs built for this specific purpose and measures only selenium compounds which are included in pathways of "real" selenoproteins. The bio-assay of the inven-
tion is suitable for the determination of selenium in blood and also other biological materials and has several advantages over procedures currently used for this purpose.

**BRIEF DESCRIPTION OF THE INVENTION**

The invention relates to plasmids carrying a selenium-specifying DNA sequence of the *E. coli* fdnF gene upstream the *E. coli* lacZ gene which permits the incorporation of selenocysteine into β-galactosidase.

More particularly, the plasmids according to the invention comprise at their beginning a lacZ derivative carrying at least the -9 to +47 nucleotide bases of the TGA codon region of *E. coli* fdnF gene.

Preferred plasmids according to the invention comprise at their beginning a lacZ derivative consisting of nucleotide bases -9 to +47 or -1 to +47 of the TGA codon region of *E. coli* fdnF gene.

Especially preferred are plasmids pRM2 deposited at the ATCC under No. 75594 and pRM4 deposited at the ATCC under No. 75595.

In a further aspect the invention relates to microorganisms transformed with a plasmid according to the invention, having selenium-dependent β-galactosidase activity, *E. coli* being preferred.

Furthermore, the invention provides a method for quantitative determination of selenium in selenium derivatives in a biological sample comprising incubating transformed microorganisms according to the invention in a suitable medium.
also containing said sample and measuring the level of β-galactosidase activity.

The method of the invention may further comprise a preliminary step in which said biological sample is subjected to treatment with acid vapor prior to being added to incubation medium.

The method according to the invention is particularly suitable for the determination of selenium in biologically active selenium derivatives and can be employed for the determination of selenium derivative in blood samples, food samples and in other biological materials.

Additionally, the invention relates to a diagnostic kit for the quantitative determination of selenium in selenium derivatives in accordance with the method of the invention.

DESCRIPTION OF THE FIGURES
Fig. 1 The synthesis of a selenium-dependent fused protein directed by plasmids pRM2 and pRM4
E. coli strains MC4100 (A and C) and its selC derivative RM1 (B and D) were transformed by plasmids pMR1 (slot 1), pRM2 (slot 2), pRM4 (slot 3), and pMR1 (TGAC) (slot 4).
Freshly transformed cells were grown in M9 minimal medium, labeled either with $[^{35}\text{S}]$-methionine (A and B) or with $[^{75}\text{Se}]$-selenite (C and D). The labeled cells were lysed and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels which were subsequently autoradiographed. Prior to electrophoresis, the $[^{35}\text{S}]$-methionine labeled lysates were treated with antibodies against β-galactosidase and immuno-precipitated. For details, see Materials and Methods.
The position of the 140kD fused protein product of genes λcI-lac'I"Z is indicated in the gel by an arrow.

(E) The TGA containing segment of the *E. coli* fdhF gene present in plasmid pRM2 (nucleotides -1 to +47) and pRM4 (nucleotides -9 to +47). Here the segment is shown as an RNA molecule in its secondary structure according to Zinoni et al. [(1990) ibid.]. The UGA codon is boxed.

Fig. 2 The selenium-dependent synthesis of β-galactosidase directed by plasmids pRM2 and pRM4 in *E. coli*. 
*E. coli* strain MC4100 and its selC derivative RM1 (A) and strain YN3230 and its selC derivative RM2 (B) were transformed by each of the plasmids pMR1, pRM2, pRM4 and pRM1 (TGAC). Freshly transformed cells were grown in M9 medium to mid-log phase in the absence or presence of sodium selenite (final concentration 1μM/ml). Levels of β-galactosidase activity were determined as described previously [Kopelowitz (1992) ibid., for details see Materials and Methods]. The results represent at least 3 independent experiments. The figures for percent of β-galactosidase activity have been normalized taking the activity directed by plasmid pMR1 as 100%.

Fig. 3 Determining the concentrations of several simple selenium-containing compounds: The bio-assay (A-D) versus Atomic Absorption Spectrophotmetry (E). 
*E. coli* strain YN3230 (+) and its selC derivative RM2 (- -) were transformed by pRM4. The transformed cells were grown to mid-log phase in M9 medium in the presence of various concentrations of selenite 3 (A), selenocysteine (B), selenate (C), and selenomethio-
nine (D). The levels of β-galactosidase activity are presented in Miller Units and were determined as described in Fig. 2. The results represent the average of 4 experiments.
In Fig. 3E, concentrations of the selenium-containing derivatives (as indicated) were determined by Atomic Absorption Spectrophotometry (AAS) using the Varian graphite furnace SpectrAA 300 Zeeman Atomic Spectrophotometer. This Spectrophotometer has an automatic background correcting system; a solution of 0.1% nitric acid and palladium was used as a chemical modifier (10 µl of 500 µg/ml). SeO₂ (c[Se]=1.000 ± 0.002 g/l) was used as a standard selenium solution. For the final estimate for each sample, the mean values for three sequential injections of aliquots were used.

Fig. 4 The effect of HCl-vapor treatment on simple Se derivatives used for the bio-assay.
The assay described in Fig. 3 was used to determine levels of selenite (A), selenocysteine (B), selenate (C), and selenomethionine (D) which were applied either directly to E. coli strain YN3230 carrying the SelC gene (-- □ --), or after HCl-vapor treatment to either strain YN3230 (--■--) or to its SelC derivative (-- ○ --). The selC E. coli derivative RM2 was used as a control. The Se-containing compounds (selenite, selenocysteine, selenate, and selenomethionine) were used either directly or after HCl-vapor-hydrolysis treatment. Only compounds treated with HCl vapor were used with the selC derivative RM2 control strain. (E) Determination of Se concentration in several Se-containing compounds by AAS, carried out as in Fig. 3.
Fig. 5 Se status in blood serum.
Selenium status in blood serum samples of rats fed [Se(+)] or [Se(-)] diets was determined by the present bio-assay, AAS and GPX activity.
A: Determination by the bio-assay as described in Fig. 3. 50-100 µl blood serum samples were subjected to HCl-vapor hydrolysis prior to the assay. Before determining Se levels by AAS, the blood serum samples were diluted 1/40 to prevent viscosity.
B: GPX activity was quantitated by the coupled enzyme procedure of Lawrence and Burk [Biochem. Biophys. Res. Comm. (1976) 71, 952-958].
The results of the measurements by the bio-assay, AAS and GPX each represent the average of three independent experiments.

DETAILED DESCRIPTION OF THE INVENTION
The present invention relates to a specific and sensitive bio-assay for the quantitative determination of selenium in selenium derivatives in biological materials, to transformed micro-organisms to be used therewith and to plasmids for generating these transformed microorganisms.

Recombinant DNA technology has been used to provide reporter systems than can be used generally to detect cis-acting elements like regulatory sites or trans-acting elements like proteins, RNA, and other large biological molecules. This approach has now been used in a new way to measure the presence and concentration of the essential chemical trace element selenium.

The present invention is based on the finding that selenium is incorporated into polypeptides in the form of
selenocysteine; and ii) in **E. coli** this incorporation is permitted by the presence of a TGA codon within a specific codon context [Zinoni et al. (1990) ibid.; Heider et al. (1992) ibid.]. Such a selenium-specific TGA codon context is present in the **E. coli** fdhF gene which specifies for the selenium-containing enzyme formate dehydrogenase [Zinoni et al. (1990) ibid.]. These selenium-specifying sequences of the fdhF gene were inserted upstream from the **E. coli** lac'Z gene at the junction of the artificially fused genes lac'Z located on plasmids pRM2 and pRM4 (Table 1B).

In *in vivo* experiments, plasmids carrying the inserted fdhF sequences directed selenium incorporation into a fused polypeptide product (Figs. 1A and 1C) which has β-galactosidase activity that is selenium-dependent (Fig. 2). Since neither the fused protein nor β-galactosidase activity are obtained in selC derivatives lacking the gene for tRNA^{Sec}^<\text{Ecoli}> it is suggested that selenium is incorporated in the form of selenocysteine (Figs. 1B, 1D and 2). Furthermore, the level of β-galactosidase is proportionally and specifically related to the simple selenium derivatives selenite and selenocysteine (Figs. 3A and 3B). Thus, the present system of plasmids in appropriate **E. coli** strains can be used as a bio-assay for determining the selenium concentrations in these compounds. Either plasmid pRM2 or plasmid pRM4 can be used. However, plasmid pRM4 is preferred since it directs slightly higher levels of the selenium-dependent β-galactosidase activity (Fig. 2). This is probably because, unlike pRM2, pRM4 carries the nine additional nucleotides which precede the 47 nucleotides following the TGA of **E. coli** gene fdhF (see Table 1B).

The invention therefore relates to plasmids carrying a selenium-specifying DNA sequence of the **E. coli** fdhF gene
upstream the *E. coli* lac'Z gene which permits the incorporation of selenocysteine into β-galactosidase.

Specific plasmids according to the invention are those comprising at their beginning a lacZ derivative carrying at least the –9 to +47 nucleotide bases of the TGA codon region of *E. coli* fdhF gene.

A preferred plasmid according to the invention is a plasmid comprising at its beginning a lacZ derivative consisting of nucleotide bases –9 to +47 of the TGA codon region of *E. coli* fdhF gene.

Another preferred plasmid comprises at its beginning a lacZ derivative consisting of nucleotide bases –1 to +47 of the TGA codon region of *E. coli* fdhF gene.

Most preferred are plasmids pRM2 deposited at the ATCC under No. 75594 pRM4 deposited at the ATCC under No. 75595.

In a further aspect the invention relates to microorganisms transformed with a plasmid according to the invention, having selenium-dependent β-galactosidase activity, *E. coli* strains being preferred.

Particularly preferred micro-organisms are *E. coli* strains transformed with plasmid pRM2 or with plasmid pRM4.

Additionally, the invention relates to a method for the quantitative determination of selenium in selenium derivatives in a biological sample comprising incubating transformed micro-organisms according to the invention in a suitable medium also containing said sample and measuring the level of β-galactosidase activity.
In preferred embodiments the method according to the invention employs transformed E. coli strains.

The method according to the invention is particularly suitable for the determination of biologically active selenium derivatives, for example sodium selenite or selenocysteine.

By the method according to the invention selenium derivatives can be quantitatively assayed different biological materials such as blood, food, and others. For assaying samples which contain relatively large amounts of protein, particularly blood samples, it is preferable to first subject the sample to acid-vapor treatment, under which proteins undergo hydrolysis.

Thus, in a preferred embodiment for assaying selenium in protein-containing samples, particularly blood samples, the sample is first subjected to acid-vapor treatment, and are then incubated with transformed micro-organisms according to the invention in a suitable medium in accordance with the method of the invention, followed by measuring the level of β-galactosidase activity.

In a preferred embodiment HCl vapor is used.

A diagnostic kit for the quantitative determination of selenium derivatives, employing the transformed micro-organisms and methods of the invention is also within the scope of this application.

The assays currently available for determining selenium concentrations in various compounds are mainly physical or chemical methods by which the element selenium is determined directly [Ihnat et al. (1986) ibid.; Neve (1991) ibid.].
Since one of the best methods for this purpose is direct Atomic Absorption Spectrophotometry, the atomic absorption of selenium in various simple selenium derivatives including sodium selenite, sodium selenate, selenocysteine, and selenomethionine, using selenium dioxide solutions as a standard (Fig. 3E) was measured. As is shown in the following examples, the atomic absorption method can be used to measure the selenium concentrations in each of these compounds, and in the range of 2-100 ng Se/ml the results are linear and are similar for all the compounds. In comparison to this physical method, the bio-assay according to the invention, which reflects UGA directed selenium incorporation, is more specific because it responds to only a few simple selenium derivatives. Probably because sodium selenite (Fig. 3A) and selenocysteine (Fig. 3B) can penetrate the cells and are included in the biochemical pathway of E. coli UGA-directed selenocysteine incorporation, the levels of β-galactosidase activity are a linear function of low concentrations of these compounds. On the other hand, because of these same criteria, the bio-assay cannot be used to measure selenium in either sodium selenate (Fig. 3C) or selenomethionine (Fig. 3D). In addition, the bio-assay is sensitive to sodium selenite and selenocysteine at concentrations (0.07-1.0 ng Se/ml) (Fig. 3A) about 40 times lower than those can be measured by Atomic Absorption Spectrophotometry (2-100 ng Se/ml) (Fig. 3E).

As described here, the bio-assay of the invention is suitable for determining selenium concentrations either in the inorganic form selenite or in the simple organic form selenocysteine. Bacteria cannot use selenium when it is in complicated organic forms. For example, in order to use the bio-assay to measure the selenium in proteins like GPX, 5' deiodinase or selenoprotein P, the protein must first be
converted to a simple inorganic or organic form by proteolysis. Thus, the basic method of the invention may be modified such that samples are first subjected to acid-vapor treatment, conditions known to cause hydrolysis in proteins. Fig. 4 shows the effects of acid treatment of various Se-containing compounds on their ability to stimulate the expression of the Se-reporter system. The levels of βGal were found to be linearly proportional to the concentrations of sodium selenite, sodium selenate, or selenocysteine in the sample solutions.

The bio-assay according to the invention has several advantages over currently available physical assays. The physical assays require sophisticated instruments not always available in clinical laboratories. The present recombinant DNA bio-assay, preferably carried out in E. coli, is simple and relatively inexpensive. In addition, at least for sodium selenite and selenocysteine, the present bio-assay is sensitive at lower concentrations than is Atomic Absorption Spectrophotometry (Fig. 3E), one of the best available physical methods. A major advantage of the bio-assay of the invention may be that, unlike the physical methods, it is specific only for certain simple selenium derivatives like selenite or selenocysteine. Measuring the total selenium status of a biological sample (like blood) may be misleading. For example, the physical methods available (Fig. 3E) are also sensitive to selenium when it is in the form of selenomethionine which is incorporated into proteins as a random substitute for methionine [Cowie, D.B. et al. (1957) Biochem. Biophys. Acta 26, 252-261; Sliwkowski, M.X. and Stadtman, T.C. (1985) J. Biol. Chem. 260, 3140-3144; Frank, P. et al. (1985) J. Biol. Chem. 260, 5518-5525; Beilstein, M.A. and Whanger, P.D. (1986) J. Inorg. Biochem. 33, 31-46], unlike selenocysteine which, directed by a UGA-codon, is
incorporated into the active sites of specific selenoproteins like GPX. Thus, in relation to human health, using a bio-assay limited to measuring biologically active forms of selenium would be preferable than using unrestricted physical methods. Therefore, the new selenium bio-assay of the invention, restricted to reflecting the UGA directed incorporation of selenocysteine, appears to be a better indicator for studies on the relation of selenium and human health than other assays currently available, since it measures specific selenium compounds included in the pathways of "real" selenoproteins.

The modified bio-assay was tested by using it to determine the Se status in samples of rat blood serum. The results of the bio-assay were compared with measurements by two well established methods, Atomic Absorption Spectrophotometry and the level of the activity of the Se-containing enzyme glutathione peroxidase (GPX). Blood serum samples from two groups of rats: (i) Rats fed their usual diet [Se(+)] and (ii) Rats fed a Se-deficient diet [Se(-)] for a 6 week period was used. The bio-assay was used to determine the Se status after the serum samples were subjected to acid hydrolysis (Fig. 5). As shown in Fig. 5A, in the [Se(+)] group, the levels of Se in the sera were 290 ng/ml according to AAS and 240 ng/ml according to the Se bio-assay. The level of Se in the sera of the [Se(-)] group was so drastically reduced that it could not even be detected by AAS. However, our the bio-assay of the invention was sensitive enough to detect Se at the low level of 4ng/ml, barely 2% of the amount of Se in the sera of the [Se(+)] group. Measured by GPX activity (Fig. 5B), 640 units were found in the sera of the [Se(+)] group and 23 units, or 3.6%, in the sera [Se(-)] group. Thus, the relative amounts of Se found in the sera of the two experimental groups were
comparable when measured by the bio-assay as a reflection of GPX activity.

The growing implications of selenium in nutrition and medicine support the need for an adequate means of assessing the selenium concentrations in biological materials [Neve (1991) ibid.]. The present recombinant DNA bio-assays is both an alternative and an addition to the physical methods currently available.

The invention will be described in more detail on hand of the following examples.
EXAMPLES

MATERIALS AND METHODS

Materials

\(^{35}\text{S}\)-methionine (>800 Ci/m mole) and the sodium salt \(^{75}\text{Se}\)-selenite (350 mCi/m mole) (1 Ci = 37 GBq) were obtained from Amersham (England). Monoclonal antibodies to \(\beta\)-galactosidase were purchased from Promega (USA). Sodium selenite, sodium selenate, selenomethionine, and selenocystine were obtained from Sigma (USA). Selenocystine was converted to selenocysteine by the addition of approximately 50 times more dithiothreitol (DTT) (Sigma, USA) and incubation at 37°C for 2 hrs; selenocysteine generation was confirmed by HPLC. Selenium dioxide (\(\text{SeO}_2\)) in \(\text{HNO}_3\), 0.5 mol/l was obtained from Merck (Germany). Palladium atomic absorption standard solution was obtained from Sigma (USA).

Media

Bacteria were grown in LB or in M9 minimal medium [Miller (1972) ibid.] (pH 7.0) supplemented with 10 \(\mu\text{M} \text{Na}_2\text{MoO}_4\) and a mixture of amino acids each at a final concentration of 20 \(\mu\text{g/ ml}\). The final concentration of cysteine was 200 \(\mu\text{g/ ml}\). Unless otherwise stated, methionine was missing from the media. All the reagents added to M9 medium were analytically pure, and the media were prepared in water double distilled in a Corning water purification system. Ampicillin (100 \(\mu\text{g/ ml}\)) was added to media in which the plasmid carrying strains were grown.

Bacterial strains and plasmid derivatives

The \textit{E. coli} strains and the plasmids used in this study are listed in Table 1-A.
### Table 1-A

**E. coli Strains**

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<td>MC41000</td>
<td>Δ(argF-lac) rpsL150</td>
<td>CGSC** (36)*</td>
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<td>WL81460</td>
<td>Derivative of MC4100 Δ(srl-reCA)305::Tn10 Δ(selC400::Kan)</td>
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<td>Derivative of MC4100 Δ(selC400::Kan)</td>
<td>P1 transduction of Δ(selC400::Kan from strain WL81460</td>
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<td>YN3230</td>
<td>Derivative of MC4100 TetR prfB1</td>
<td>(24,28)*</td>
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<tr>
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<td>Derivative of YN3230 Δ(selC400::Kan)</td>
<td>P1 transduction of (selC400::Kan from strain WL81460</td>
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</table>

*(19) - Zinoni et al. (1990) ibid.
(28) - Kawakami et al. (1988) ibid.
**E. coli Genetic Stock Center, Yale University
### Table 1-B

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMR1</td>
<td>A pBR322 derivative carrying the fused genes λcI'-lac'I&quot;Z</td>
</tr>
<tr>
<td>pMR1(TGAC)</td>
<td>A pMR1 derivative carrying a TGA codon followed by a C residue at the junction of λcI'-lac'I&quot;Z.</td>
</tr>
<tr>
<td>pRM2</td>
<td>A pMR1 derivative carrying the TGA codon region of E. coli gene fdhF (nucleotides -1 to +47) at the λcI-lac'I&quot;Z junction, at the beginning of the lacZ gene.</td>
</tr>
<tr>
<td>pRM4</td>
<td>Like pRM2 except that the TGA region of the E. coli fdhF gene includes nucleotides -9 to +47</td>
</tr>
<tr>
<td>pFM</td>
<td>A pACYC184 derivative carrying the E. coli fdhF gene.</td>
</tr>
</tbody>
</table>


*(13) - Zinoni et al. (1986) ibid.*
Plasmid pFM20 carries the *E. coli* fdhF [Zinoni et al. (1986) ibid.]. Using the required primers and with plasmid pFM20 as a template, PCR technique was used to amplify two overlapping regions of the fdhF gene flanked by restriction sites HindIII and BamHI: i) the DNA from nucleotides -1 to +47 (plasmid pRM2); and ii) the DNA from nucleotides -9 to +47 (plasmid pRM4). The nucleotides are numbered relative to the TGA codon of gene fdhF and the positions are indicated by arrows in Fig. 1E.

Molecular cloning
All the recombinant DNA manipulations were carried out by standard procedures [Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY)]. Restriction enzymes and other enzymes used in the recombinant DNA experiments were obtained from New England Biolabs (USA). DNA sequencing was carried out using the sequenase kit of United States Biochemicals (USA).

Bacterial growth and transformations, and measurements of β-galactosidase activity
*E. coli* cells were transformed [Sambrook (1989) ibid.] by the plasmid of choice (see Table 1B). Single colonies of freshly transformed cells were grown on LB plates for 6-8 hrs and then overnight in M9 medium. On the following day, they were diluted in M9 medium to which a selenium containing derivative was added; the cells were then grown at 37°C for 2-3 hrs to mid-log phase (OD$_{600}$ = 0.4-0.6). The cells were examined for β-galactosidase activity in culture aliquots treated with SDS as described by Miller [Miller (1972) ibid.].
Labeling and identification of the in vivo synthesis of the fused gene product \( \lambda cI' - lac' I'' \)

Freshly transformed cells were grown in M9 medium at 37°C overnight in the presence of either 1.0 Ci/ml \(^{75}\text{Se}\)-selenite or 15 μCi/ml \(^{35}\text{S}\)-methionine. During labeling with \(^{75}\text{Se}\)-selenite, cold sodium selenite was added to a final concentration of 1.5 μM. Cells were lysed and proteins were extracted and then immunoprecipitated with antibodies to β-galactosidase as described previously [Schoulaker-Schwartz, R. et al. (1991) Proc. Natl. Acad. Sci. USA 88, 4996-4500]. Cell lysis and protein extraction were carried out in the presence of 0.2mM PMSF. Proteins were separated on 7.5% polyacrylamide gels by electrophoresis and detected by autoradiography.

HCl-vapor treatment

Samples to be treated with HCl vapor were transferred to a 6 x 50mm Pyrex tube, dried by vacuum, and sealed into a large vessel to which 6N HCl had been added at the bottom. This procedure permits the acid vapor only to be in contact with the sample. Vapor-phase was carried out under vacuum at 110°C for 48 h. The dried samples were suspended in M9 minimal medium and added to the bacterial culture.

Preparation of sera

Weanling Sprague-Dawley rats (40-50g) were obtained from Charles River Laboratories (Wilmington, MA). For each experimental procedure, two groups of four rats each were fed a torula yeast-based, semi-synthetic [Se(+) or [Se(-)] diet (Teklad Premier, Madison, WI). The Se deficient [Se(-)] diet (product #TD86298) contained 0.016 mg Se/kg; the Se replete [Se(+)] control diet (product #TD87177) had the same formulation but was supplemented with 0.1 mg Se/kg in the form of sodium selenite (Na₂SeO₃). After six weeks, sera
were prepared from blood samples taken from all four rats of each group. Care of the rats and all experimental protocols were done in accordance with the appropriate institutional guidelines and the approval of the Animal Review Committee of Dartmouth Medical School, Lebanon, NH.

GPX activity

GPX activity was quantitated by the coupled enzyme procedure of Lawrence and Burk [ibid.]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7) 1 mM EDTA/1 mM NaN₃/1 mM GSH/ and 1 E.U./ml glutathione reductase per ml. The serum sample (0.1 ml) was added to 0.8 ml of reaction mixture and pre-incubated for 5 min at 25°C before the reaction was initiated by the addition of 0.1 ml H₂O₂ (2.5 mM). Absorbance at 340 nm was recorded for 5 min. One unit of activity catalyzes the oxidation of 1.0 µmol of NADH reduced per min. In order to correct for background, distilled water was used instead of the sample.

RESULTS

A reporter system for detecting UGA-directed selenocysteine incorporation into a polypeptide

Recombinant DNA technology had been previously used by the present inventors to construct a reporter system for sensitively detecting UGA readthrough by tryptophanyl-tRNATrp in E. coli [Kopelowitz (1992) ibid.]. The present system has been constructed for detecting UGA readthrough by selenocysteinyl-tRNASEcULp. For this purpose plasmid pMR1 which carries the fused genes λcl'-lac'I'Z [Kopelowitz (1992) ibid.; Gray (1982) ibid.] was used. At the junction of these genes, regions surrounding the TGA codon of the E. coli fdhF gene were inserted; these sequences have been found to permit selenocysteine incorporation into polypeptides [Zinoni (1990) ibid.]. The plasmid carrying the region of
fdhF from -1 to +47 is called pRM2, and that carrying region -9 to +47 is called pRM4 (and see Materials and Methods and Fig. 1E).

In the *E. coli* MC4100 derivatives examined, the fused genes Δci'-lac'I"Z of pMR1 direct the synthesis of a 140kD polypeptide. This polypeptide is labeled by [35S]-methionine (slot 1 of Fig. 1A and 1B), but not by [75Se]-selenite (slot 1 of Figs. 1C and 1D). On the other hand, in strain MC4100, the protein product of the gene fusions on plasmids pRM2 and pRM4 is labeled by [75Se]-selenite (Fig. 1C, slots 2 and 3). As expected, there was no radioactive label incorporated in strain RM1, a selC derivative of MC4100 (slots 2 and 3 of Figs. 1B and 1D). Thus, the radioactivity in the product of the fused genes of pRM2 and pRM4 is in the form of selenocysteine.

Release factor 2 (RF2) competes with natural or mutated UGA suppressors for the recognition of the UGA codon [Caskey, C.T. (1980) TIBS. 5, 234-237; Curran J.F. and Jarus, M. (1986) Proc. Natl. Acad. Sci. USA 83, 6538-6542)]; mutation prfB1 in the gene specifying for RF2 increased UGA suppression by a UGA suppressor tRNA and UGA readthrough by tRNA^{Trp} [Kopelowitz (1992) ibid.; Kawakami, K. et al. (1988) J. Bactriol. 170, 5378-5381; Roesser, J.R. et al. (1989) J. Biol. Chem. 264, 12284-12288]. To check whether the prfB1 mutation increase the level of UGA-directed selenocysteine incorporation, a derivative of *E. coli* strain MC4100, called YN3230, that carries a prfB1 mutation [Kawakami (1988) ibid.] was included in the experiment. By labeling with [75Se]-selenite or [35Se]-selenite or [35S]-methionine it was found that this mutation had no effect on the UGA-directed selenocysteine incorporation into the Δci'-lac'I"Z product of either pRM2 or pRM4 (data not shown). However,
quantitative measurements of β-galactosidase activity levels revealed that the prfb1 mutation did slightly affect the selenium-dependent synthesis of the protein fusion products of both plasmids prM2 and prM4 (Fig. 2). As shown in Fig. 2, the level of the synthesis of β-galactosidase is selenium independent when directed by pMR1, which has no TGA codon at the cI-lac'I"Z junction. In strain MC4100 the levels of β-galactosidase directed by prM2 was 22% and by prM4 was 24% (Fig. 2A); in strain YN3230 that was directed by prM2 was 25% and that by prM4 was 31% (Fig. 2B). These values include 2% selenium-independent β-galactosidase synthesis which is directed by either prM2 or prM4 in strains MC4100 and YN3230 in the absence of selenium or in the selC derivatives of these strains in the presence of selenium (Fig. 2). Experimental work was continued with plasmid prM4 in strain YN3230 where the highest levels of selenium-dependent β-galactosidase activity was found.

The bio-assay for determining the presence and concentration of selenium in various simple selenium derivatives. Finally, the selenium-dependent β-galactosidase activity directed by the gene fusion cI-lac'I"Z of prM4 in YN3230 was examined as to whether it could be used as a measure for determining the concentrations of simple selenium derivatives in solutions. As shown in Fig. 3, the levels of β-galactosidase are linearly related to the concentrations of both sodium selenite and selenocysteine. For sodium selenite, linearity is in the range of 0.06-1.0 ng Se/ml (Fig. 3A) and for selenocysteine in the range of 2-30 ngSe/ml (Fig. 3B). However, for sodium selenate and selenomethionine this is not the case. Levels of β-galactosidase activity are a function of the logarithm of the concentration of sodium selenate only at very high concentrations ($10^3$-$10^5$ ngSe/ml) (Fig. 3C); no β-galactosidase activity at all was found in
the presence of selenomethionine with (data not shown) or without added methionine (Fig. 3D).

For comparison, the concentration of selenium using each of the described derivatives by the well known Atomic Absorption Spectrophotometric procedure was also determined. As shown (Fig. 3E), for each of the derivatives studied, in the range of 2-100ng Se/ml absorption was a linear function of the concentration of selenium in the compound.

**Acid treatment**

Bacteria cannot use Se when it is incorporated in complicated organic forms, as it is in blood proteins. To overcome this limitation, the above-described bio-assay of the invention may be modified so that samples are first subjected to acid-vapor treatment, conditions known to cause hydrolysis in proteins. Fig. 4 shows the effects of acid treatment of various Se-containing compounds on their ability to stimulate the expression of the Se-reporter system. The levels of βGal are found to be linearly proportional to the concentrations of sodium selenite, sodium selenate, or selenocysteine in the sample solutions. As already mentioned, the response of the basic bio-assay to these compounds is also dependent on the presence of the E. coli SelC gene (Fig. 4A-4C) which specifies for tRNA^Sec [Leinfelder, W. et al., Nature (1988) 331, 723-725]. Furthermore, for each of these three Se derivatives, the linearity of the response is in the range of 0.05-1.0 ng Se/ml (Figs. 4A-4C). These values are about 20 times lower than the range of sensitivity of the method of AAS (Fig. 4E). In addition, in the case of sodium selenate the linearity of the βGal activity is in the range of 0.05-1.0 ng Se/ml, but only after the samples are subjected to acid conditions. This is probably because selenate, which
presumably neither penetrates *E. coli* cells nor is utilized by them, is converted under the acidic conditions to selenite, which does penetrate the cells and is included in the UGA-directed selenocysteine pathway. It may be noted that without the acid-vapor step the bio-assay is not at all sensitive to selenomethionine, which is not included in the UGA directed selenocysteine pathway, even after acid hydrolysis treatment (Fig. 4D).

**Blood serum assay**

The modified bio-assay was tested by using it to determine the Se status in samples of rat blood serum. The results of the bio-assay were compared with measurements by two well established methods, AAS (see above) and the level of the activity of the Se-containing enzyme GPX. Blood serum samples from two groups of rats was used: (i) Rats fed their usual diet [Se(+)]) and (ii) Rats fed a Se-deficient diet [Se(-)] for a 6 week period. The bio-assay was used to determine the Se status after the serum samples were subjected to acid hydrolysis (Fig. 5). As shown in Fig. 5A, in the [Se(+)] group, the levels of Se in the sera were 290 ng/ml according to AAS and 240 ng/ml according to the selenium bio-assay. The level of Se in the sera of the [Se(-)] group was so drastically reduced that it could not even be detected by AAS. However, the bio-assay of the invention was sensitive enough to detect Se at the low level of 4ng/ml, barely 2% of the amount of Se in the sera of the [Se(+)] group. Measured by GPX activity (Fig. 5B), 640 units in the sera of the [Se(+)] group and 23 units, or 3.6%, were found in the sera [Se(-)] group. Thus, the relative amounts of Se found in the sera of the two experimental groups were comparable when measured by the present bio-assay or as a reflection of GPX activity.
CLAIMS:

1. A plasmid carrying a selenium-specifying DNA sequence of the E. coli fdhF gene upstream the E. coli lac'Z gene which permits the incorporation of selenocysteine into β-galactosidase.

2. A plasmid according to claim 1 comprising at its beginning a lacZ derivative carrying at least the -9 to +47 nucleotide bases of the TGA codon region of E. coli fdhF gene.

3. A plasmid according to claim 2 comprising at its beginning a lacZ derivative consisting of nucleotide bases -9 to +47 of the TGA codon region of E. coli fdhF gene.

4. A plasmid according to claim 2 comprising at its beginning a lacZ derivative consisting of nucleotide bases -1 to +47 of the TGA codon region of E. coli fdhF gene.

5. Plasmid pRM2 deposited at the ATCC under No. 75594.

6. Plasmid pRM4 deposited at the ATCC under No. 75595.

7. Micro-organisms transformed with a plasmid according to any one of claim 1 to 6 having selenium-dependent β-galactosidase activity.

8. Micro-organisms according to claim 7 being E. coli.

9. E. coli transformed with plasmid pRM2.

10. E. coli transformed with plasmid pRM4.
11. A method for the quantitative determination of selenium in selenium derivatives in a biological sample comprising incubating micro-organisms according to any one of claims 7 to 10 in a suitable medium also containing said sample and measuring the level of β-galactosidase activity.

12. A method according to claim 11 wherein said micro-organism in E. coli according to claim 9.

13. A method according to claim 11 wherein said micro-organism in E. coli according to claim 10.

14. A method according to claim 11 which further comprises a preliminary step in which said biological sample is subjected to treatment with acid vapor prior to being added to incubation medium.

15. A method according to claim 15 wherein in said preliminary step said biological sample is vacuum dried prior to being subjected to said acid vapor treatment.

16. A method according to claim 14 or claim 15 wherein said acid is HCl.

17. A method according to any one of claim 11 to 16 wherein said selenium derivative is a biologically active selenium derivative.

18. A method according to claim 17 wherein said selenium derivative is a selenite.

19. A method according to claim 17 wherein said selenium derivative is selenocysteine.
20. A method according to any one of claims 14 to 19 wherein said sample is blood.

21. A method according to any one of claims 11 to 19 wherein said sample is a food sample.

22. A diagnostic kit for the quantitative determination of selenium in selenium derivatives.
## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)


Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>A</td>
<td>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 87, issued June 1990, Zinoni et al., &quot;Features of the formate dehydrogenase mRNA necessary for decoding of the UGA codon as selenocysteine&quot;, pages 4660-4664.</td>
<td>1-22</td>
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</table>

Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search: 21 NOVEMBER 1994

Date of mailing of the international search report: 12 DEC 1994

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
C12N 9/04, 9/38, 1/20, 15/00; C12Q 1/02, 1/32

A. CLASSIFICATION OF SUBJECT MATTER:
US CL:
435/190, 207, 320.1, 252.3, 7.37, 7.6, 7.9, 26, 29; 536/23.4

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.
435/190, 207, 320.1, 252.3, 7.37, 7.6, 7.9, 26, 29; 536/23.4

B. FIELDS SEARCHED
Documentation other than minimum documentation that are included in the fields searched:
NONE

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):
APS, DIALOG