Human uracil-DNA glycosylase complements *E. coli ung* mutants

Lisbeth C. Olsen, Rein Aasland, Hans E. Krokan and Dag E. Helland*
Laboratory of Biotechnology, University of Bergen—HiB, N-5020 Bergen and ¹UNIGEN Center for Molecular Biology, University of Trondheim, N-7030 Trondheim, Norway

Received May 13, 1991; Revised and Accepted July 17, 1991

ABSTRACT

We have previously isolated a cDNA encoding a human uracil-DNA glycosylase which is closely related to the bacterial and yeast enzymes. In *vitro* expression of this cDNA produced a protein with an apparent molecular weight of 34 K in agreement with the size predicted from the sequence data. The *in vitro* expressed protein exhibited uracil-DNA glycosylase activity. The close resemblance between the human and the bacterial enzyme raised the possibility that the human enzyme may be able to complement *E. coli ung* mutants. In order to test this hypothesis, the human uracil-DNA glycosylase cDNA was established in a bacterial expression vector. Expression of the human enzyme as a LacZα-humUNG fusion protein was then studied in *E. coli ung* mutants. *E. coli* cells lacking uracil-DNA glycosylase activity exhibit a weak mutator phenotype and they are permissive for growth of phages with uracil-containing DNA. Here we show that the expression of human uracil-DNA glycosylase in *E. coli* can restore the wild type phenotype of ung mutants. These results demonstrate that the evolutionary conservation of the uracil-DNA glycosylase structure is also reflected in the conservation of the mechanism for removal of uracil from DNA.

INTRODUCTION

Spontaneous deamination of bases in DNA occurs at a considerable rate in living cells and may give rise to transition mutations (1, 2). Cytosine and 5-methylcytosine deaminate to uracil and thymine respectively, resulting in U:G and T:G mispairs. Adenine deaminates to hypoxanthine resulting in an H:T basepair (usually referred to as an inosine:thymine basepair). Prokaryotic and eukaryotic organisms possess base specific DNA repair enzymes like uracil- and thymine- and hypoxanthine-DNA glycosylases. These enzymes can counteract the mutagenic effect of base deaminations by removing the deamination product and thereby initiating the base excision repair pathway (3, 4). Uracil-DNA glycosylase will also remove uracil from A:U basepairs and acts on both double and single stranded DNA (5).

A group of related genes encoding uracil-DNA glycosylases has recently been described with members in several species including bacteria, yeast, man as well as the herpes viruses (6–12). In addition to the human uracil-DNA glycosylase cDNA (*UNG15/40*) isolated by us, two other human cDNAs encoding uracil-DNA glycosylases have been reported and they are apparently unrelated to this gene family as well as to each other (13, 14). The gene encoding the latter cDNA is located on chromosome 5 (14), while the gene encoding *UNG15/40* has been assigned to chromosome 12 (15). Intriguingly, the cDNA isolated by Muller and Caradonna shows considerable sequence similarity to the conserved region of the cyclin gene family.

Mitochondria have been reported to contain a uracil-DNA glycosylase (16–18). Whether the nuclear and the mitochondrial forms of uracil-DNA glycosylase are structurally related is not known. We have recently found evidence suggesting that the *UNG15/40* gene encodes a nuclear enzyme (G. Slupphaug, L.C.O., R.A., D.E.H., and H.E.K., submitted). The mitochondrial enzyme may derive from one of the other genes mentioned above or from a different gene. As an alternative, we have proposed that the nuclear and mitochondrial form may both arise from the *UNG15/40* gene (9).

Among the characterized members of the uracil-DNA glycosylase family, the human uracil-DNA glycosylase is most similar to the *E. coli Ung* protein showing 55.7% sequence identity. A functional similarity is also supported by the fact that the PBS2 encoded uracil-DNA glycosylase inhibitor protein (*Ugi*) is capable of blocking the activity of both the bacterial and the human uracil-DNA glycosylases by binding to the enzyme (19, 20). However, *Ugi* does not inhibit other DNA glycosylases (19). These observations raised the question that human uracil-DNA glycosylase might be able to function in *E. coli*.

We decided to test this possibility by examining whether human uracil-DNA glycosylase can complement strains of *E. coli* deficient in this enzyme. Complementation of *E. coli* DNA repair

†Note: The human gene from which the UNG15 and UNG40 cDNA clones derive has been assigned the name *DGU* (15) but in order to explicitly refer to this gene, we use the term *UNG15/40* throughout this paper.

* Present address: Dana Farber Cancer Institute, Boston, MA 02115, USA
mutants tagA/alkA and ada was recently successfully used to isolate the corresponding mammalian DNA repair genes encoding 3-methyladenine-DNA glycosylase and O²-methylguanine-DNA methyltransferase (21, 22). We have taken advantage of the fact that E. coli ung mutants show a weak mutator phenotype and are permissive for growth of phages with uracil-containing DNA (23–25). By expressing the human uracil-DNA glycosylase in an E. coli ung mutant, we here show that the human gene is capable of complementing the ung-mutation.

**MATERIAL AND METHODS**

**Bacterial strains, phage strains and plasmids**

E. coli NR8051 [Δ(pro-lac), thi⁻, ara] and NR8052 [Δ(pro-lac), thi⁻, ara, trpE9777, ungI] (25) were provided by Dr. Thomas A. Kunkel, National Institute of Environmental Health Sciences, USA. E. coli CJ236 duA, ungI, thiI, relAl /pCJ105(Cm) was purchased from Bio-Rad. Phage λvir was a gift from Dr. Dietmar Kamp, Laboratory of Biotechnology, University of Bergen, Norway. The plasmid pUNG15 contains a human uracil-DNA glycosylase cDNA with the complete open reading frame, while pUNG40 contains a shorter cDNA lacking the 59 first codons (9).

**Preparation of [³H-uracil]DNA**

[³H-uracil]DNA was prepared either by nick-translation or by the polymerase chain reaction (PCR) (26). Nick-translation of calf thymus DNA in the presence of [⁵⁻³H]dUTP (Amersham) at a specific activity of 0.5 Ci/mm mol was carried out as described by Krokan and Wittwer (27). The specific activity of the nick-translation substrate was 2.9 x 10⁶ cpm/µg DNA. A 525 bp DNA fragment of known sequence was used to prepare [³H-uracil]DNA by PCR. The PCR reaction mixture contained 1 µM of the SP6 and T7 primers respectively, 0.5 mM each of dATP, dCTP and dGTP (Pharmacia), 55 µCi of [⁵⁻³H]dUTP (21 Ci/mmol; Amersham), approximately 0.2 ng of pGBS3 and 1 unit Taq polymerase (Cetus) in the PCR-buffer recommended by the manufacturer. The reaction was carried out in a volume of 50 µl. PCR was run for 30 cycles of 1 minute each at temperature 94°C, 60°C, and 72°C using a Cetus Perkin Elmer thermocycler. After 30 cycles another unit of Taq polymerase was added and PCR was continued for 30 new cycles. The DNA was then precipitated, dissolved in 50 µl 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and chromatographed on a Sephadex G-50 column. The specific activity of the PCR generated substrate was 7.4 x 10⁶ cpm/µg DNA.

**Detection of uracil-DNA glycosylase activity**

Uracil-DNA glycosylase activity was assayed at 30°C for 30 minutes in the presence of 40 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.25 µg/µl BSA and [³H-uracil]DNA (14,000 cpm when using nick translated substrate [Figure 1], and 8,000 cpm when using the PCR generated substrate [Figure 4]). Sonicated salmon sperm DNA was added as a coprecipitant to a final concentration of 0.72 µg/µl and the DNA precipitated by adding 500 µl ice cold 5% TCA. After centrifugation for 10 minutes at 12,000 g the amount of acid-soluble radioactive material was measured using a liquid scintillation counter.

**Paper chromatography**

The reaction mixture from the uracil-DNA glycosylase assay, was heat inactivated for 2 minutes at 100°C and applied to GB 002 paper (Schleicher & Schuell) and developed with isobutyric acid, ddH₂O, 0.1 M EDTA, concentrated ammonia, toluene (160:22:3:2:20 by volume) as previously described (28). The chromatogram was then cut into 2 cm pieces, and the radioactivity of each fraction determined. dUTP, uridine and uracil (all from Sigma) were included as markers.

**In vitro transcription/translation**

The plasmids pUNG15 and pUNG40 were linearized (at the 3'-end of the cDNA-insert relative to the T7 or SP6 promoter being used) by treatment with an appropriate restriction endonuclease. Standard in vitro transcription assays were performed in a final volume of 100 µl containing 2–5 µg template DNA. Synthesis of sense and antisense RNA was carried out at 37°C for 1 hour using either T7 or SP6 RNA polymerase (Promega) in accordance with the manufacturer’s protocol. Capped transcripts were synthesized by including m⁷G(S')pppS'G (Pharmacia) at 0.5 µM. Ribonucleotides were present at 0.5 µM except for rGTP which was present at a concentration of 0.05 µM. The DNA template was removed by adding RNase free DNasel (Pharmacia) and the incubation was continued at 37°C for 15 minutes. The in vitro synthesized RNA was extracted once with phenol-chloroform (1:1) and once with chloroform and ethanol precipitated.

In vitro translation of the in vitro synthesized RNA was carried out at 30°C for 30 minutes in the presence or absence of [³²S]L-methionine (1159 Ci/mmol; NEN) using a nuclease treated rabbit reticulocyte lysate (Amersham). Standard in vitro translation reactions were performed in a final volume of 50 µl containing 35 µl rabbit-reticulocyte lysate and in vitro synthesized RNA. If not otherwise stated, all RNA generated from one transcription reaction was used in each translation reaction.

When in vitro translation was performed for the subsequent analysis of the translation products by gel electrophoresis, 70 µCi of [³²S]L-methionine was included in the translation mixture. After incubation, the translation products were precipitated with acetone, resolved in 100 µl SDS-sample buffer. Aliquots of 25 µl were electrophoretically separated on a 12.5% SDS-polyacrylamide gel (29). [¹⁴C]Methylated proteins (Amersham) were used as molecular weight markers. The gel was dried and autoradiographed. Unlabelled proteins were tested for uracil-DNA glycosylase activity.

**Construction of uracil-DNA glycosylase expression plasmid**

The plasmid pG7bUNG was constructed by inserting the 1775bp BamHI-EcoRI fragment of pUNG15 (nucleotide position 297 to 2071; position no. 1 is the first nucleotide of the cDNA insert in pUNG15) into BamHI and EcoRI digested pGEM-7Zf(+) (Promega), generating a lacZα-humUNG fusion gene (Figure 3). A correct junction between lacZα and humUNG was confirmed by DNA sequencing.

**Transformation**

Transformation of competent bacteria with 1 ng DNA was performed by electroporation (30) using a Bio-Rad GenePulsor at 25 µF, 2.5 kV and the pulse controller set to 200 ohms.

**Induction of LacZα-humUNG fusion protein and preparation of cell extracts**

Overnight cultures of E. coli cells were diluted 1:100 with LB medium (50 ml final volume) supplemented with ampicillin when appropriate and grown at 37°C for 2 hours. IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) was added to a final concentration...
of 1 mM to cultures of NR8052/pG7bUNG cells and incubated for an additional 2 hours. The cells were harvested by centrifugation at 4,500 g for 15 minutes at 4°C. The pellets were resuspended in 5 ml 50 mM Tris-HCl pH 8.0, 1 mM EDTA and disrupted by sonication (5 pulses of 30 seconds) using an Ultrasonic instrument at 30 μA. Cell debris was removed by centrifugation at 12,000 g for 15 minutes at 4°C. The supernatant was diluted 1:50 in 50 mM Tris-HCl pH 8.0, 1 mM EDTA and aliquots of 2, 5, and 12 μl were assayed for uracil-DNA glycosylase activity.

Plaque assay

Passages of λvir through either E. coli NR8051 or CJ236 were performed to generate thymine containing (λvir[T]) and uracil-containing phages (λvir[U]), respectively. The ability of these two types of phages to grow on different bacterial strains was then tested.

Mutator assay

To detect the number of rifampicin resistant bacteria 1 ml of the appropriate bacterial culture was mixed with 3 ml top agarose and poured on LB-plates containing 50 μg/ml rifampicin (Sigma) and incubated overnight at 37°C. The colonies were counted and the number of rifampicin resistant colonies per 10⁸ viable cells was calculated. In the mutator assay we have estimated the smallest lethal dose of rifampicin to be 50 μg/ml.

RESULTS

In vitro expression of human uracil-DNA glycosylase cDNA clones

In vitro expression analysis of two human placental cDNA clones (9) were performed and the translation products tested for enzyme activity. Uracil-DNA glycosylase activity was easily detected after in vitro transcription/translation of the UNG15 cDNA (Figure 1). This result demonstrates that this cDNA clone encodes a functional uracil-DNA glycosylase. In vitro transcription/translation of the shorter cDNA clone, UNG40, did not give rise to any functional protein, probably due to the lack of an appropriate in-frame methionine codon. No uracil-DNA glycosylase activity was detected in the rabbit reticulocyte lysate itself (data not shown). Neither was any enzyme activity detected upon translation of UNG15 antisense RNA.

The molecular weight of the uracil-DNA glycosylase which was purified from human placenta was estimated to be 29 K (31). The N-terminal amino acid sequence of this enzyme was the basis

Figure 1. Uracil-DNA glycosylase activity of in vitro expressed human UNG-cDNA clones. Sense and antisense RNA transcripts were generated from UNG-cDNA clones using T7 or SP6 RNA polymerase, and the transcripts were translated using a rabbit reticulocyte lysate. Calf thymus [3H-uracil]DNA was used as substrate in this experiment.

Figure 2. SDS-PAGE of in vitro expressed human uracil-DNA glycosylase cDNA. Translation products from reactions where no RNA (lane 1), all (lane 2), or 50% (lane 3) of the RNA generated from one in vitro transcription reaction was added to the translation reactions. Lane S: 14C-labelled molecular weight markers. The arrow indicates the position of the in vitro synthesized 34 K polypeptide.

Figure 3. a) Schematic drawing of the expression vector pG7bUNG. The construction of this vector is described in Material and Methods. The smallest filled arrow followed by the open box indicates the lac-promoter, the accompanying lacZ-region and the remaining part of the polylinker of pGEM-7Zf(+). The light shaded box labelled pre and the following dark shaded arrow labelled UNG represent the coding region from the human uracil-DNA glycosylase cDNA; pre indicates the portion of the cDNA encoding the last 14 amino acids of the putative presequence. The thin open box indicates the 3'-untranslated region of the cDNA. T7 indicates the position of the T7 promoter. b) Sequence of the joint between the LacZa-peptide reading frame and the humUNG-reading frame.
for the isolation of UNG40 and UNG15 cDNAs. The open reading frame of the UNG15 cDNA clone has a coding capacity for a protein of molecular weight of 33.8 K (9). Radiolabelled in vitro translation-products were analyzed by SDS-PAGE and visualized by autoradiography. A polypeptide with an apparent molecular weight of 34 K was observed in agreement with the size estimated from the sequence data (Figure 2).

Expression of human uracil-DNA glycosylase cDNA in E. coli

The strong amino acid sequence similarity between the human and bacterial uracil-DNA glycosylases suggests that the human uracil-DNA glycosylase cDNA may complement the ung mutation in E. coli. To test this hypothesis, the expression vector pG7bUNG was constructed, where the 63 N-terminal codons of the UNG-reading frame were replaced with the 26 N-terminal codons of the lacZ-reading frame of pGEM-7Zf(+) (Figure 3). This LacZα-humUNG fusion protein lacks most of the putative presequence of the human uracil-DNA glycosylase (9). The lactZα-humUNG fusion gene is under control of the inducible lac-promoter. The ung− bacterial strain NR8052 was then transformed with this plasmid.

Lysates of NR8052 and NR8052/pG7bUNG as well as the isogenic ung+ strain, NR8051, were tested for uracil-DNA glycosylase activity (Figure 4). The enzyme activity of NR8052/pG7bUNG was similar to that of the wild type strain NR8051, while the ung mutant strain NR8052 showed less that 1% of the activity of the wild type strain. These results demonstrate that the human uracil-DNA glycosylase is expressed as a functional enzyme in E. coli. Extracts from a NR8052 strain carrying vector without insert, pGEM-7Zf(+), did not reveal any detectable uracil-DNA glycosylase. Treatment of NR8052/pG7bUNG with the lac-inducer IPTG did not consistently result in higher levels of enzyme activity as compared to untreated cells. The expression of the LacZα-humUNG fusion protein in the absence of inducer is probably due to the titration of the lac-repressor as a result of the high copy number of plasmids in these cells. An inhibition of the enzyme apparently occurs when the largest amounts of bacterial extracts were assayed (Figure 4; NR8051 and NR8051/pG7bUNG + IPTG).

Released tritiated material from DNA incubated with cell extracts of NR8052/pG7bUNG comigrated with uracil in paper chromatography. Cell extracts from the ung− strain did not release any detectable material comigrating with uracil (data not shown). These results show that the liberated tritiated products in the standard enzyme assay resulted from a uracil-DNA glycosylase activity and not from unspecific nucleases.

Human uracil-DNA glycosylase is able to remove uracil from U:A basepairs in E. coli

The presence of an active human uracil-DNA glycosylase in lysates of NR8052/pG7bUNG does not imply that the human enzyme is capable of complementing E. coli ung mutants in vivo. Two different biological assays have been used to examine if the human uracil-DNA glycosylase gene product may restore the wild-type phenotype of E. coli ung mutant cells. These assays are based on the facts that E. coli ung mutants exhibit a weak mutator phenotype (23) as well as being permissive for phages with uracil-containing DNA (24, 25).

Uracil-containing phages were prepared by passing λvir through an E. coli dut ung strain where as much as 30% of the thymine content may be replaced by uracil (32). These λvir[U] phages are severely restricted in their ability to multiply in uracil-DNA glycosylase expressing bacteria since the phage DNA is degraded by the combined action of the glycosylase and AP-endonucleases. The phage assay measures the ability of the human enzyme to remove uracil from an A:U basepair.

The ability of λvir[U] to grow on NR8052/pG7bUNG was reduced with a factor of about 350 as compared to NR8052 (Figure 5), indicating that λvir[U] is fragmented into smaller pieces due to an active human uracil-DNA glycosylase working together with AP endonucleases. The titer of λvir[U] grown on uninduced NR8052/pG7bUNG was 16 fold higher as compared to the induced NR8052/pG7bUNG. This indicates that IPTG has a positive effect on the expression of the LacZα-humUNG fusion protein under these conditions. No difference in the plating efficiency of λvir[U] on NR8052/pGEM-7Zf(+) compared to NR8052 was seen (Figure 5). The titer of λvir[U] on NR8052/pG7bUNG was 30 fold higher as compared to the wild-type cells, suggesting that there is a difference in the ability of the human enzyme to remove U in an A:U basepair as compared to the bacterial enzyme. Longterm storage of NR8052/pG7bUNG resulted in bacteria that were more permissive for growth of λvir[U] as compared to cells that have been transformed with pG7bUNG immediately before running the phage assay. This may be due loss or alteration of the plasmid during storage.

Human uracil-DNA glycosylase is able to remove uracil from U:G mismatches in E. coli

Spontaneous mutation to rifampicin resistance due to C:G—T:A transition mutations in the rpoB gene has been reported to be about 5-fold higher in ung mutants then in wild type cells (23). Different E. coli strains were grown on LB-plates containing rifampicin and the number of resistant colonies scored. The
wild type cells. These results clearly indicate that expression of strains used to estimate the statistical significance of the differences between pairs of viable cells as determined for individual plates. The student West was EPTG and rifampicin, the number of revertants was reduced 1.8 fold higher in NR8052 ung~ cells as compared to wild-type cells (Figure 6). When NR8052/pG7bUNG cells were grown in the presence of both IPTG and rifampicin, the number of revertants was reduced 1.8 fold as compared with uninduced NR8052/pG7bUNG cells and wild type cells. These results clearly indicate that expression of the human uracil-DNA glycosylase in E. coli ung mutants suppresses the weak mutator phenotype of these cells.

DISCUSSION

In this report, we have shown that a human uracil-DNA glycosylase cDNA gave rise to an active gene product both after in vitro expression and by expression in E. coli ung mutant cells.

The molecular weight of the in vitro expressed human uracil-DNA glycosylase was found to be 34 K in agreement with the molecular weight estimated from the open reading frame of the UNG15 cDNA. However, the molecular weight of the purified protein from human placenta was reported to be 29 K (31) and the reported N-terminal amino acid sequence of this protein is situated at position 78–105 in the predicted sequence of the UNG15 encoded protein (9). Based on this observation we suggested that the human uracil-DNA glycosylase may be processed in vivo (9). Alternatively, the smaller size of the purified human placental uracil-DNA glycosylase may be a result of artificial cleavage during the purification procedure. A similar situation is found in yeast, where the purified uracil-DNA glycosylase is smaller than predicted from sequence data (8).

The protein expressed from the UNG15 cDNA in vitro is similar in size to the 33–37 K mammalian uracil-DNA glycosylases previously reported (33–35). However, the apparent structural differences between UNG15 and the cDNAs described by Vollberg et al., (13) and Muller and Caradonna (14) indicates that the encoded proteins are different.

We have earlier reported that uracil-DNA glycosylases from different species including man and E. coli are highly conserved (9). This observation suggested that the human uracil-DNA glycosylase gene may complement E. coli ung deficient cells. Here we have shown that expression of the human protein as a LacZα-humUNG fusion protein in E. coli ung~ cells almost completely restored the wildtype phenotype of these mutant cells. The activity of the LacZα-humUNG fusion protein in the bacteria depends on the efficiency of transcription and translation, as well as the catalytic efficiency of the enzyme in the bacteria. Since we have not examined these aspects of expression of human uracil-DNA glycosylase in E. coli, we cannot conclude from our present data that the human and the bacterial enzymes have comparable specific activities in the bacteria.

The phage assay and the mutator assay clearly demonstrated that the human enzyme when expressed in E. coli, is capable of removing uracil from both U:A and U:G basepairs. These data indicates that the repair mechanism involved in the removal of uracil from DNA in E. coli and human cells are quite similar. However, it should be noted that the complementation appears complete in the mutator assay (Figure 6) while only partial complementation was observed with the phage assay (Figure 5). This result can be explained by the fact that the 26 N-terminal amino acids corresponding to the LacZα-peptide of the fusion protein may disturb the catalytic efficiency of the fusion protein or may indicate that the human enzyme has a different specificity towards U:G and U:A basepairs as compared with the E. coli enzyme. Alternatively, these observations may be explained by the anticipated presence of a high concentration of uracil in the phage DNA as compared to a much lower concentration of uracil in the chromosomal DNA due to cytosine deamination in the mutator assay.

The mammalian DNA repair enzymes 3-methyladenine-DNA
glycosylase and O6-methylguanine-DNA methyltransferase have recently been cloned by phenotypic rescue of DNA repair-deficient E. coli mutants (21, 22). These results together with our complementation data indicate that elements of several DNA repair pathways are evolutionarily conserved between prokaryotes and mammals. These data further suggest that other eukaryotic DNA repair genes may be isolated by phenotypic rescue of E. coli mutants. A partial complementation has indeed been observed by expression of the rat DNA polymerase β in E. coli polA mutants (36). Using complementation as a strategy for isolation of DNA repair genes is also encouraged by the recent success in the isolation of a series of mammalian genes by complementation of mammalian cells which are deficient in DNA repair (37).

In experiments using monoclonal antibodies, Seal and Sirover (38) found that uracil-DNA glycosylase and DNA polymerase α may be physically associated. It has thus been speculated that uracil-DNA glycosylase may be organized in a multiprotein DNA repair complex. If uracil-DNA glycosylase must be part of a multiprotein complex in order to be fully functional, one would expect that complementation of E. coli ung− cells by addition of only the human UNG gene would not occur since the human uracil-DNA glycosylase would probably be unable to recognize other bacterial proteins involved in such a multiprotein complex. Relevant to these considerations is the recent report that DNA polymerase δ cannot be replaced by the phage T4 DNA polymerase alone in an in vitro SV40 replication assay. However, the DNA polymerase δ with its accessory proteins (PCNA and polymerase alone in an in vitro & polymerase) may be physically associated. It has thus been speculated that DNA repair (37).

ACKNOWLEDGEMENTS

We thank Dr. Dietmar Kamp and Dr. Erling Seeberg for advice and helpful discussions and Dr. Rune Male for his comments on the manuscript. This work was supported by grants from the Norwegian Cancer Society, the Norwegian Society for Science and the Humanities, and Lise and Arnfinn Hejes fond. L.C. Olsen holds a research fellowship at the Department of Biochemistry, University of Bergen.

REFERENCES