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De novo amplification within a "silent" human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides

(butyrylcholinesterase/inheritable gene amplification/*in situ* hybridization/parathion exposure/silent CHE phenotype)

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ABSTRACT **A 100-fold DNA amplification in the *CHE* gene, coding for serum butyrylcholinesterase (BtChoEase), was found in a farmer expressing the "silent" CHE phenotype. Individuals homozygous for this gene display a defective serum BtChoEase and are particularly vulnerable to poisoning by agricultural organophosphorous insecticides, to which all members of this family had long been exposed. DNA blot hybridization with regional BtChoEase cDNA probes suggested that the amplification was most intense in regions encoding central sequences within BtChoEase cDNA, whereas distal sequences were amplified to a much lower extent. This is in agreement with the "onion skin" model, based on amplification of genes in cultured cells and primary tumors. The amplification was absent in the grandparents but present at the same extent in one of their sons and in a grandson, with similar DNA blot hybridization patterns. *In situ* hybridization experiments localized the amplified sequences to the long arm of chromosome 3, close to the site where we previously mapped the *CHE* gene. Altogether, these observations suggest that the initial amplification event occurred early in embryogenesis, spermatogenesis, or oogenesis, where the *CHE* gene is intensely active and where cholinergic functioning was indicated to be physiologically necessary. Our findings demonstrate a *de novo* amplification in apparently healthy individuals within an autosomal gene producing a target protein to an inhibitor. Its occurrence in two generations from a family under prolonged exposure to parathion indicates that organophosphorous poisons may be implicated in previously unforeseen long-term ecological effects.**

Mammalian cell cultures and tumor tissues often acquire resistance to drugs or inhibitors by selection of cells with inheritable amplified genes encoding the target protein of these agents (1, 2). Well-known examples are the amplified dihydrofolate reductase (1) and Na^+,K^+-ATPase (3) genes, conferring resistance to methotrexate or ouabain, respectively. Numerous DNA amplification events were found in primary and metastatic tumors and in established cell lines but not in whole animals or in humans.

The ubiquitous enzyme butyrylcholinesterase (BtChoEase; acylcholine acylhydrolase, EC 3.1.1.8) is capable of hydrolyzing the neurotransmitter acetylcholine and is specifically inhibited by organophosphorous poisons (4), including the agricultural insecticide parathion (*p*-nitrophenyl diethyl thionophosphate) (5). Individuals expressing the "silent" BtChoEase phenotype produce a defective enzyme (6) with a different amino acid sequence (7), rendering them particularly sensitive to parathion (8). The silent BtChoEase phenotype is generally attributed to a single point mutation in the gene coding for serum BtChoEase (5–8), although the exact nature of this mutation has not been described. High frequency of defective BtChoEase phenotypes (9) and high incidence of chronic organophosphate poisoning (10) were both reported in Israel. However, the molecular consequence of this combination could not be investigated due to lack of cloned BtChoEase cDNA probes. Recently, we have cloned the human BtChoEase cDNA (11) and mapped the *CHE* genes, coding for this enzyme, to chromosomes 3 and 16 (12). By using regional cDNA probes from BtChoEase cDNA (13), we initiated a search for alterations in the *CHE* genes in individuals expressing the silent BtChoEase phenotype and exposed to organophosphates.

Intact cholinergic functioning appears to be important in germ-line cell development and early embryogenesis: various cholinergic elements (14, 15), and particularly BtChoEase (16), are expressed early in embryogenesis, cholinergic inhibitors block sperm motility (17), and high levels of BtChoEase mRNA are already present in developing human oocytes (18). This makes the *CHE* gene a good candidate for amplification *in vivo*, under conditions where the germ-line cells are exposed to organophosphate poisoning and particularly in cases of silent homozygotes, which are more sensitive to such exposure. Here, we report on a *de novo* 100-fold amplification of a genomic fragment hybridizing with BtChoEase cDNA and localized on chromosome 3.

The amplification occurred in an individual under prolonged exposure to organophosphorous insecticides who displays the silent serum BtChoEase phenotype. A similar amplification was found in a son of this individual. These findings present one example of an amplification within an autosomal gene in apparently healthy individuals and imply that the frequent use of organophosphorous poisons may have long-term inheritable consequences on humans.

MATERIALS AND METHODS

Measurements of serum BtChoEase activities were performed spectrophotometrically by the acetylthiocholine hydrolysis technique (19). Tetraisopropyl pyrophosphoramide (iso-OMPA) (20) and 2-butoxy-*N*-(2-diethylaminoethyl)-4-quinoline carboxamide (dibucaine) (21) were used for specific inhibition tests, discriminating the silent serum BtChoEase phenotype. DNA blot hybridization experiments were performed as described (11), using peripheral blood DNA and

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Abbreviations: BtChoEase, butyrylcholinesterase; iso-OMPA, tetraisopropyl pyrophosphoramide.

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regional BtChoEase cDNA probes (13). *In situ* hybridization to spread metaphase lymphocyte chromosomes was carried out as detailed elsewhere (12).

RESULTS

The occurrence of the silent BtChoEase phenotype in the H family was brought to our attention when one of its members, I.T., suffered from characteristic prolonged apnea (22) following a single intravenous administration of succinylcholine during the course of general anesthesia performed because of ongoing infertility. On another occasion, a sibling, M.I., fainted while spraying parathion (*p*-nitrophenyl diethyl thionophosphate) in a cotton field. Examination of serum BtChoEase activity in all members of this family (Fig. 1, Table 1) revealed very low levels of butyrylcholine hydrolyzing activity in serum samples from both I.T. and M.I., with increased sensitivity to the specific organophosphorous BtChoEase inhibitor iso-OMPA (5, 20) and pronounced resistance to the local anesthetic dibucaine, all being characteristic of the atypic BtChoEase type enzyme (23).

To examine whether the expression of the silent BtChoEase phenotype was due to alteration(s) at the level of the *CHE* genes, DNA blot hybridization experiments were performed, using ^{32}P-labeled fragments from the cloned BtChoEase cDNA (11, 13) as probes. When digested with enzymes *Eco*RI and *Hin*dIII and probed with full-length BtChoEase cDNA, peripheral blood DNA from M.I. revealed highly positive restriction fragments of ≈6.0 kilobases (kb) and smaller (for *Eco*RI) and ≈2.5 kb (for *Hin*dIII). These were absent from DNA from several other members of the H family, specifically including the grandparents, R.U. and M.O., and the sibling, I.T. (Fig. 2). This pattern was reproducibly obtained using DNA samples taken at 6-month time intervals. Interestingly, the hybridization signal with these amplified fragments was considerably weaker when the 5′ and 3′ terminal parts of BtChoEase cDNA were used as probes, suggesting that the amplification event was confined mainly to the central part of the BtChoEase gene and that the external parts of this gene were amplified to a lesser extent, in agreement with the "onion skin" model, described for other amplification units, serving as an early intermediate in the amplification process (1, 2). R.U., mother of I.T. and M.I., appeared in this analysis to have a different size *Eco*RI band than her son, M.I., and her husband, M.O. This apparent polymorphism could be observed with probes a and b (Fig. 2). Other chromosome 3 genes, such as the transferrin receptor gene and the *RAF* oncogene, were not amplified, as was demonstrated by DNA blot hybridization experiments (not shown). Furthermore, DNA from several other individuals with the silent BtChoEase phenotype, from unrelated families and occupations, did not display this amplification. To quantitate the amplification in M.I.'s DNA, a dot blot hybridization was performed with six dilutions of peripheral blood DNA from each member of the H family. DNA from M.I. and one of his sons, O.F., contained an equivalent of ≈25 pg of BtChoEase cDNA-positive sequences per μg of genomic DNA, whereas DNA from other members of the family displayed levels equivalent to only 1–3 pg/μg of DNA (Fig. 3). Blot hybridization of the very small amount of DNA that was available to us from the 2-year-old O.F. revealed restriction patterns similar to those of M.I. (not shown).

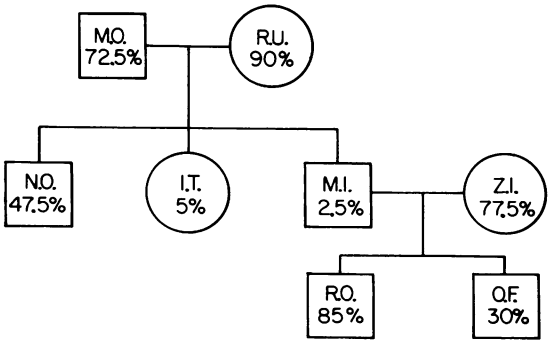

FIG. 1. The H family pedigree. The percentage of residual serum BtChoEase activity in the presence of 0.01 mM iso-OMPA as compared with the parallel activity measured in control serum in the presence of the same concentration of inhibitor (Table 1) is under each set of initials.

Table 1. Serum BtChoEase activity in members of the H family

Sample no.	Family member	BtChoEase activity*	Dibucaine IC_{50}†	iso-OMPA inhibition‡
1	R.U.	5.2 ± 0.6	1.0	31
2	M.O.	4.3 ± 0.5	7.5	31
3	N.O.	3.5 ± 0.4	10.0	45
4	I.T.	1.1 ± 0.2	50.0	78
5	M.I.	0.8 ± 0.1	100.0	82
6	Z.I.	5.1 ± 0.2	10.0	36
7	R.O.	4.9 ± 0.3	10.0	31
8	O.F.	2.1 ± 0.4	10.0	44
9	Control§	6.6 ± 1.3	6.3	38

*BtChoEase activity, in μmol of thiocholine released per ml of serum per min, was determined spectrophotometrically by the acetylthiocholine hydrolysis technique (19). Assays were performed in multiwell plates and several time points were measured in a Bio-Tek EL-309 microplate reader. Spontaneous release of thiocholine was subtracted and rates of enzymatic activity were calculated from linear regression curves of optical density at 405 nm. Values are presented as mean ± SEM from three independent measurements of three different serum samples for each person. Time intervals between samplings were at least 6 months.

†Dibucaine inhibition is expressed as the μM concentration of inhibitor that blocks 50% of thiocholine release under the assay conditions detailed in footnote *.

‡The percentage of thiocholine release that was inhibited by increasing the concentration of the organophosphorous inhibitor iso-OMPA (2) from 0.1 μM to 0.01 mM is presented.

§Control serum was a pooled mixture of equal volumes of serum samples from 10 apparently healthy individuals with normal BtChoEase activities. Note the high level of thiocholine release, the low IC_{50} values for dibucaine, and the moderate iso-OMPA inhibition for control serum as compared with samples 4 and 5.

When metaphase peripheral blood chromosomes from M.I. and his mother, R.U., were analyzed by the Giemsa (G) (25) and the bromodeoxyuracil-induced (R) (26) banding techniques, apparently normal karyotypes were observed in both individuals, with neither minute chromosomes nor homogeneously stained regions that are commonly found in cases of gene amplifications (1, 2). *In situ* hybridization with ^{35}S-labeled BtChoEase cDNA revealed intense labeling of the 3q29 region in M.I.'s chromosomes as compared with controls (Fig. 4). ^{35}S labeling was mostly confined to chromosomal structures, excluding the possibility that the amplified *CHE* genes were present in submicroscopic extrachromosomal elements (27). Altogether, these observations indicated that the inheritable amplified DNA segment was localized close to the original site of the *CHE* gene at 3q26 (12).

In spite of the apparent gene amplification in M.I. and O.F., gel electrophoresis and immunoblot analysis of serum proteins with anti-BtChoEase antibodies failed to reveal overexpression of serum BtChoEase in these individuals. The antibodies employed were (*i*) Dakko's commercial rabbit antibody against human serum BtChoEase and (*ii*) a rabbit antiserum elicited in our laboratory against clone-produced BtChoEase peptides prepared using a bacterial expression vector (28). Both of these antibodies reacted positively with

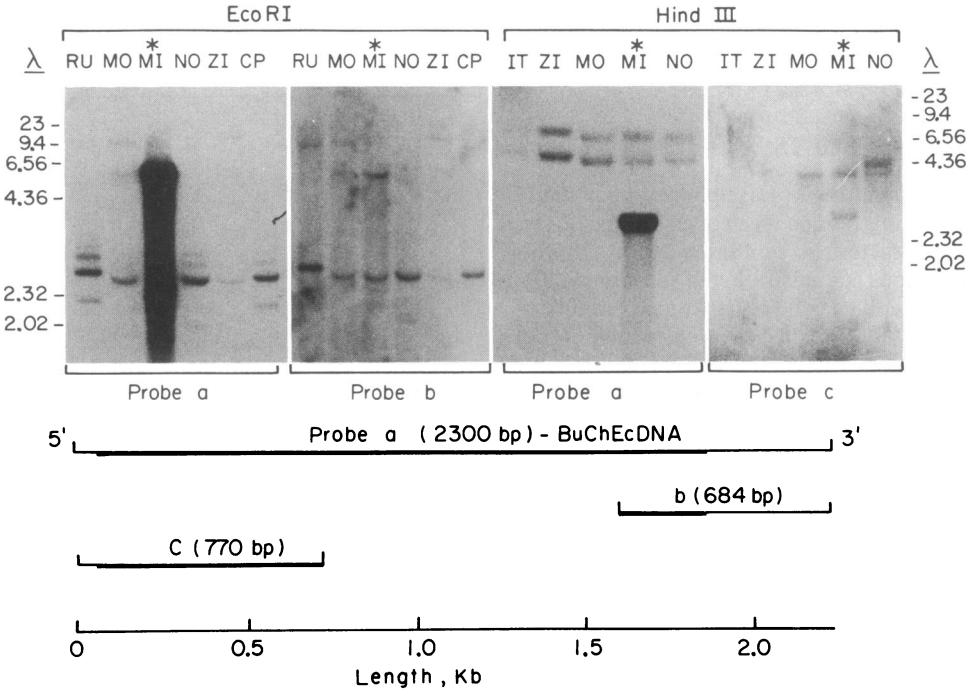

FIG. 2. DNA blot hybridization with regional BtChoEase cDNA probes. Ten-microgram samples of DNA from peripheral blood were digested to completion with excess amounts of restriction enzyme *Eco*RI (Pharmacia) or *Hin*dIII (IBI), electrophoresed on 1.0% agarose gel, and transferred to a GeneScreen filter (NEN). Subcloned BtChoEase (BuChE) cDNA probes a, b, and c were prepared as described (11, 13) and labeled with [^{32}P]dATP by the random primer technique (24). Hybridization was carried out for 16 hr and filters were washed in 0.15 M NaCl/0.015 M sodium citrate/0.1% sodium dodecyl sulfate at 60°C. Exposure was for 6 days at −70°C with an intensifying screen. λ phage DNA digested with the restriction enzyme *Hin*dIII served for size markers. The lanes loaded with DNA from M.I., the individual who suffered from parathion poisoning, are marked with an asterisk. The probes used are schematically drawn below, with coding regions represented by thick lines and untranslated regions represented by thinner lines. Note that the labeled cDNA probes light up the prominent 2.0- to 2.5-kb fragments in genomic DNA from the parents, siblings, and spouse of M.I., in agreement with previous results (11, 13). The relatively weak 4.7- and 9.4-kb genomic DNA fragments that also hybridize with this probe (11, 13) can hardly be seen under these exposure conditions. In contrast, DNA from M.I. reveals strong hybridization signals with *Eco*RI-cut DNA fragments of variable sizes. bp, Base pairs.

a serum protein of about similar intensity in all samples (not shown).

DISCUSSION

The discovery of a gene amplification in apparently healthy individuals opens several questions of considerable scientific and medical importance. The variable sizes of the positive DNA fragments obtained with *Eco*RI but not with *Hin*dIII argue for the onion skin structure for the initial event of the amplification. Furthermore, the similar blot hybridization patterns that were observed with O.F. and M.I.'s DNA indicate that the initial amplification event was inheritable. Although an onion skin DNA structure can serve as an intermediate in the production of tandem repeats, it cannot be replicated and retain its amplified conformation. Because the amplified BtChoEase DNA appears to be present in many cells of the affected individuals and to be genetically inherited, it must be there in a replicated, tandemly duplicated form not as an onion skin. Altogether, these hybridization experiments suggest that the *de novo* amplification event occurred in the genome of M.I. very early in embryogenesis, spermatogenesis, or oogenesis, rendering it inheritable. Spermatogenesis seems to be the most likely suggestion, in view of the similar *Eco*RI restriction pattern in M.I. and his father, M.O., and the apparent difference in the *Eco*RI bands observed in the DNA of the mother, R.U. In addition, sperm cells are subjected to critical developmental stages in the adult, which makes them better candidates than oocytes for the amplification and selection events. However, we cannot rule out the possibility that O.F. inherited the predisposition for this amplification and that the occurrence of the amplified DNA in his case developed during embryogenesis and was due to the prolonged exposure of M.I. to parathion.

The *in situ* hybridization observations demonstrated that the inheritable amplified DNA segment was localized close to one of the original sites of the *CHE* gene at 3q26 (12). The second site, on chromosome 16, remained apparently unaltered. This is in good agreement with the evidence of the chromosome 16 BtChoEase gene being inactive in about 92% of the Caucasian population (12). Also, it agrees with genetic linkage analysis correlating the silent mutation with chromosome 3 genes such as transferrin (reviewed in ref. 13). Since the amplification unit corresponded primarily to the middle third of the cDNA, and assuming that it is present at a single site in the genome, we calculate that at least 100 copies of the amplified fragment are present in a possibly fully inheritable form in the genomic DNA of M.I. A likely explanation for our finding that primarily the central part of the BtChoEase gene is amplified in these individuals is that this part of the gene became amplified as an inheritable tandem repeat. We could not detect overexpression of serum BtChoEase in either M.I. or O.F.'s blood. However, this does not exclude the possibility that the amplified gene was expressed early during development—for example, in germ-line cells and embryonic tissues, where the *CHE* gene is intensely expressed (14, 16, 18). It should be noted, however, that the question of how such an amplified gene might be transcribed has not been addressed in the present study.

The role of BtChoEase in embryonic cells is totally unknown, although implication to growth and cell division has been suggested (13, 16). This could possibly indicate that the amplification of the *CHE* gene might have given a growth advantage to the M.I. embryo, similar to amplified oncogenes

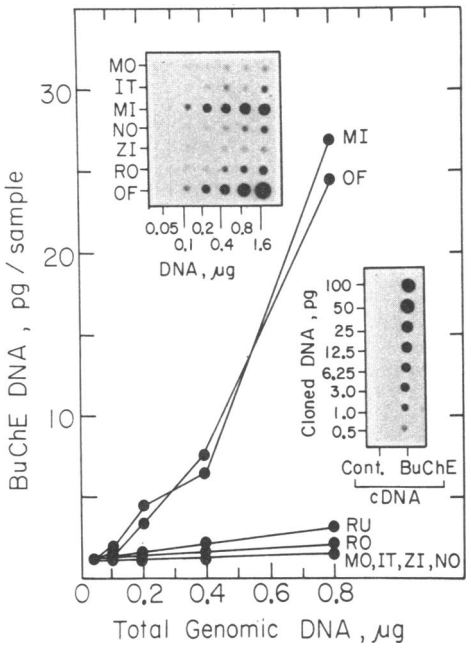

FIG. 3. Quantification of amplified *CHE* genes in members of the H family by dot blot hybridization. Denatured genomic DNA from peripheral blood cells was spotted onto a GeneScreen filter using a dot blot applicator (Bio-Rad). Electroeluted BtChoEase (BuChE) cDNA (probe a, Fig. 2) was spotted in parallel for calibration. All samples contained the noted quantities of genomic DNA and denatured herring testes DNA to yield a total amount of 2 μg of DNA per spot. Hybridization, washing, and exposure were done with ^{32}P-labeled probe a, as detailed in the legend of Fig. 2. Quantities of genomic BtChoEase DNA sequences that hybridized with the labeled probe in each member of the H family were determined in values equivalent to pg of BtChoEase cDNA by optical densitometry of the exposed x-ray film (Agfa Gevaert) in a Bio-Tek microplate reader. An irrelevant clone served as control. (*Insets*) Autoradiographed films showing dot hybridizations of μg of genomic DNA (upper) and pg of cloned DNA (lower). Cont., control.

in tumors (2). However, the nucleotide sequence of the BtChoEase cDNA does not resemble any of the known oncogenes or growth factors. The most likely explanation for the amplification event is, therefore, related with the acetylcholine hydrolyzing activity of BtChoEase.

In an otherwise normal embryo, overproduction of normal BtChoEase might interfere with cholinergic function and be lethal to the developing embryo. In contrast, overexpressed silent BtChoEase would be less harmful due to its very low catalytic activity, while improving the resistance of a developing sperm cell, oocyte, or embryo to organophosphate poisoning. We have found that both R.U. and M.O. were working in agriculture when M.I. was conceived, being exposed to high levels of parathion. When combined with the occurrence of a silent *CHE* gene, such exposure may have created conditions under which only the amplification and overproduction of silent BtChoEase would permit survival. This event could be related to the extent of exposure, perhaps explaining why I.T., also expressing the silent phenotype, does not carry the amplification, similar to several other apparently unexposed individuals with the silent phenotype.

It will be interesting to find out whether the amplification of the silent human *CHE* gene is a unique phenomenon, particularly since organophosphorous poisons are recently being exploited as commonly used insecticides and as war agents. In view of the overexpression of BtChoEase that we observed in brain tumors (29) and the altered properties of BtChoEase in the serum of carcinoma patients (30), it is possible that the normal *CHE* gene could also be subject to

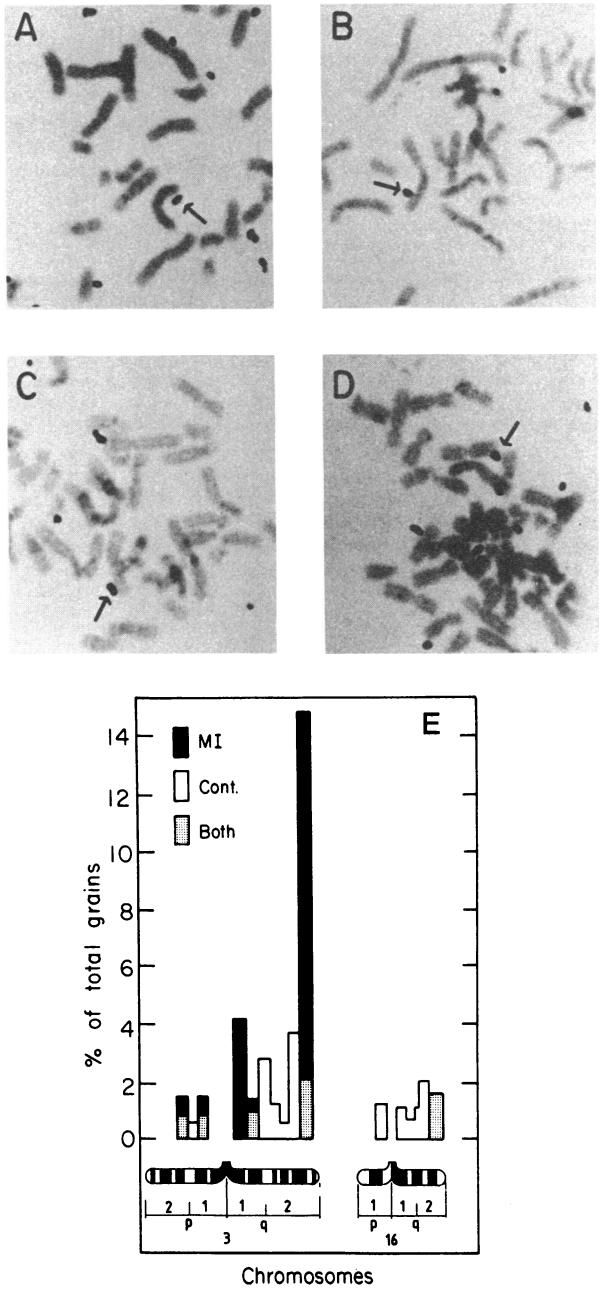

FIG. 4. Chromosomal mapping of amplified *CHE* gene by *in situ* hybridization. (*A–D*) BtChoEase cDNA hybridizes with chromosome 3. Lymphocyte metaphase chromosomes from M.I. (see Fig. 1) were employed for *in situ* hybridization with ^{35}S-labeled probe as recently described (12). Exposure was for 2–4 days under NTB-2 emulsion (Kodak) diluted 1:1 with H_2O, and development was for 30 sec with Kodak HC-110 developer. Four representative R-banded (26) partial spreads are displayed (*A–D*), in all of which the q arm of chromosome 3 is labeled (see arrows). Because of the three-dimensional structure of the chromosomes covered with the photographic emulsion, disintegrations and grain formation occur more frequently close to the chromosome than directly on it, where the emulsion layer tends to be thinner (see also ref. 12 for comparison). (*E*) Comparative mapping of amplified and normal *CHE* genes on chromosomes 3 and 16. Distribution of silver grains scored over chromosomes 3 and 16 from 21 R-banded metaphase spreads prepared from M.I.'s peripheral blood revealed that a relatively high percentage of grains was located on the 3q27-ter region, with a prominent peak on 3q29. Parallel mapping using R.U.'s chromosomes revealed a main peak on 3q26, similar to our previous mapping (Cont.) (12). Labeling on chromosome 16 was essentially similar in M.I., R.U., and control.

DNA amplification events. DNA transfection and transgenic mice experiments aimed to examine this point have already been initiated. Finally, it would be important to determine whether the silent phenotype is causedly related with ongoing infertility problems, whether repetitive exposure to organophosphates (31) provides a selective pressure for gene amplification at the *CHE* locus, and whether other commonly used chemical agents have similar effects on additional loci in man and other species.

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