Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 4147–4150, October 1975
Medical Sciences

Radioimmune determination of hypoxanthine phosphoribosyltransferase crossreacting material in erythrocytes of Lesch–Nyhan patients

(genetic disease/purine salvage enzyme/ ^{125}I iodination)

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Communicated by Esmond E. Snell, June 2, 1975

ABSTRACT **We have developed a sensitive radioimmunoassay capable of detecting and quantitating 20 ng of hypoxanthine phosphoribosyltransferase (EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) protein. For this assay, hypoxanthine phosphoribosyltransferase from human erythrocytes was iodinated with ^{125}I under mild conditions using hydrogen peroxide and lactoperoxidase attached to Sepharose-4B. Antisera prepared against homogeneous human hypoxanthine phosphoribosyltransferase precipitates the iodinated enzyme as effectively as the unlabeled enzyme. The radioimmunoassay has been used to look for hypoxanthine phosphoribosyltransferase crossreacting material in hemolysates from sixteen different patients with a marked genetic deficiency of this enzyme characteristic of the Lesch–Nyhan syndrome. Fifteen hemolysates contained no detectable (less than 1% of normal) crossreacting material. One hemolysate contained a normal amount of crossreacting material. Hypoxanthine phosphoribosyltransferase from this patient (E.S.) has been shown to be a K_m mutant enzyme [McDonald, J. A. & Kelley, W. N. (1971) *Science*, 171, 689–691].**

The Lesch–Nyhan syndrome is an X-linked genetic disorder of nucleic acid metabolism. The syndrome is characterized by mental retardation, self-mutilation, and high levels of uric acid in the blood and the urine (1). Patients with Lesch–Nyhan syndrome usually display virtual absence of activity of the purine salvage enzyme, hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) in their erythrocytes (2). Earlier investigators have concluded that erythrocytes from Lesch–Nyhan patients contained normal amounts of catalytically-nonfunctional HPRT proteins which crossreacted with antisera prepared against erythrocyte HPRT from normal individuals (3–7). Our previous work (8, 9) suggests that the HPRT preparations used to elicit antibody in some of these earlier studies may have contained other proteins besides HPRT. Therefore, it was of interest to reexamine Lesch–Nyhan hemolysates† with antibody prepared against apparently homogeneous HPRT.

We have purified HPRT from human erythocytes to apparent homogeneity and prepared monospecific antibody to the pure enzyme. The pure HPRT was labeled with ^{125}I, and a sensitive radioimmunoassay has been developed to detect HPRT protein. We have used this assay to screen hemolysates for HPRT crossreacting material.

Abbreviation: HPRT, hypoxanthine phosphoribosyltransferase.

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† We use the term "Lesch–Nyhan hemolysate" to designate hemolysates from patients with a marked genetic deficiency of HPRT, but not necessarily displaying all the symptoms of the Lesch–Nyhan Syndrome.

HPRT crossreacting materials were determined on thirty hemolysate samples in a "blind" analysis. When the data were correlated, it was found that of 16 different Lesch–Nyhan hemolysate samples, only one patient had a normal amount of HPRT crossreacting material. HPRT from this patient (E.S.) has previously been shown to be a K_m mutant (10). In contrast to previous studies, the hemolysates from the 15 other patients had less than 1% of the HPRT protein found in normal individuals.

MATERIALS AND METHODS

Blood Samples. Outdated normal blood was obtained from a blood bank. Blood erythrocytes were washed and lysed as described by Arnold and Kelley (11). Hemolysates from sixteen different patients, (D.G., M.W., D.C., J.F., H.D., E.S., V.P., M.G., J.K., J.S., W.E., R.F., Mark D., Mike D., B.D., and R.G.) and one normal subject were provided by Dr. W. N. Kelley as 30 numbered samples.

Enzyme and Enzyme Assays. The purification of HPRT has been described (9). The enzyme assays were performed as described earlier (8) except [^{14}C]hypoxanthine was used to assay enzyme activity with ^{125}I-labeled HPRT.

Preparation of Hypoxanthine Phosphoribosyltransferase Antibody. A goat was immunized by multiple injections of homogeneous human erythrocyte HPRT (along with Freund's complete adjuvant when HPRT was injected subcutaneously): on day 1 (100 μg subcutaneous), day 17 (100 μg subcutaneous), and day 25 (100 μg intravenous). On day 29 the goat was bled and the blood was allowed to clot overnight at 4°. Debris was removed from the serum by centrifugation at 20,000 × *g* for 30 min. The same goat was given additional injections of HPRT on day 48 (100 μg subcutaneous), day 62 (100 μg subcutaneous), and day 67 (100 μg intravenous). On day 74 the goat serum was prepared as described above and stored at −20°. This second serum was used for all studies in this paper. Preimmune serum, obtained by bleeding the same goat prior to injection with HPRT, was treated in an identical manner. The protein concentration in the serum was 80–100 mg/ml.

Rabbit Anti-Goat γ-Globulin Antibody. Rabbit anti-goat γ-globulin antiserum with high titer was purchased from Antibodies, Inc., Davis, Calif., and stored under liquid nitrogen.

Preparation of Lactoperoxidase-Sepharose. Sepharose-4B (Pharmacia) was activated with CNBr according to the procedure of Cuatrecasas (12). Lactoperoxidase (Sigma) was coupled to the activated Sepharose at a final concentration of 2 mg of enzyme per ml of settled beads. After the cou-

pling procedure any remaining reactive groups were inactivated with glycine (13). The Sepharose-bound lactoperoxidase was stored at 0°–4° in buffer containing 20 mM Tris·HCl, pH 7.8, 20 mM KCl, 6 mM $MgCl_2$, and 0.1 mM EDTA.

Iodination of Hypoxanthine Phosphoribosyltransferase. Iodination of HPRT was carried out at 4° by modifying the procedure of David (13) as follows: 20 μl of settled lactoperoxidase-Sepharose beads were added to 200 μg of HPRT in 60 μl of enzyme buffer (20 mM Tris·HCl, pH 7.8, 20 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol). Then, 2 μl of KI (0.01 M) were added followed by Na ^{125}I (10 mCi, carrier-free, Schwarz/Mann) in a final volume of approximately 100 μl. The iodination was initiated by adding 2 μl of a solution containing 0.1% H_2O_2. The reaction mixture was shaken gently to keep the lactoperoxidase-Sepharose in suspension. After 10 min, the entire reaction mixture was layered on top of a column (1 × 25 cm) containing Sephadex G-25 equilibrated in enzyme buffer. The column was washed with enzyme buffer and 0.3- to 0.4-ml fractions were collected. Fractions were analyzed for total radioactivity and for trichloroacetic acid precipitable radioactivity. To determine acid-precipitable radioactivity, an aliquot was added to 1 ml of solution containing 0.5 mg of bovine serum albumin. Trichloroacetic acid was added to a concentration of 10%. After chilling on ice for 1 hr the precipitate was recovered on glass fiber filters and the radioactivity was determined in a gamma counter (>80% counting efficiency). The labeled enzyme, identified by acid-precipitable radioactivity, eluted before the unreacted Na^{125}I. Twenty microliters of 0.5 M KI were added to the pooled enzyme fractions (1.7 ml) and the mixture was dialyzed against enzyme buffer. The purified ^{125}I-labeled HPRT was stored in a liquid nitrogen freezer. Bovine serum albumin (0.5 mg/ml) was added to a part of the enzyme. The iodinated enzyme retained over 75% enzyme activity. The ^{125}I-labeled HPRT initially yielded 300 cpm/ng, and due to ^{125}I decay, between 100 and 150 cpm/ng for most experiments reported in this paper.

Immunoprecipitation of Hypoxanthine Phosphoribosyltransferase. Immunoprecipitations of HPRT with goat anti-HPRT serum were carried out in 1.7-ml plastic micro-test tubes. The immunoprecipitation mixture of 100 μl contained ^{125}I-labeled HPRT (or unlabeled HPRT), goat anti-HPRT serum, rabbit anti-goat γ-globulin serum, and sufficient preimmune goat serum to keep the total goat globulin concentration constant. First, the ^{125}I-labeled HPRT enzyme (30–100 ng, about 10,000 cpm) in enzyme buffer containing 0.25% Triton X-100 and 0.25% sodium deoxycholate was mixed with goat anti-HPRT serum and/or preimmune goat serum, and the mixture was incubated at 4° for 5–8 hr. Rabbit anti-goat γ-globulin serum, sufficient to precipitate all the goat γ-globulin, was added and the mixture was incubated at 4° overnight. The precipitate was sedimented at 20,000 rpm for 10 min at 4°. In some experiments aliquots from the supernatant were withdrawn to determine enzyme activity. The precipitate was washed with 1.0 ml of enzyme buffer containing Triton X-100 and sodium deoxycholate. The supernatant was removed with a pasteur pipette and the radioactivity of the precipitate was determined in a gamma counter.

Radioimmunoassays. Competition radioimmunoassays were performed with constant concentrations of ^{125}I-labeled HPRT, goat anti-HPRT serum, and rabbit antigoat γ-globulin serum, and varying concentrations of unlabeled HPRT or hemolysate. The ^{125}I-labeled HPRT and competing samples were mixed together before the goat anti-HPRT serum was added. Otherwise, the immunoprecipitation was conducted as described above. The percent ^{125}I-labeled HPRT precipitated was calculated by assigning 100% to the amount precipitated in the absence of competing unlabeled HPRT.

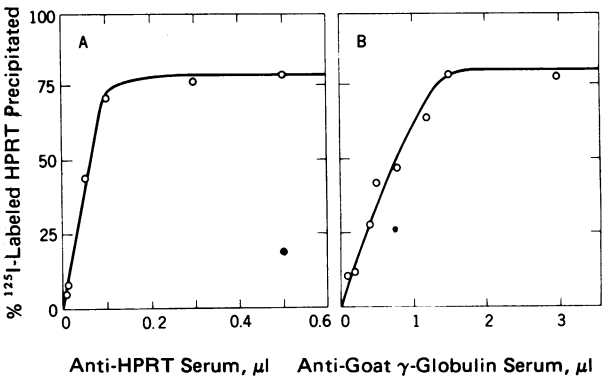

FIG. 1. (A) Precipitation of ^{125}I-labeled HPRT by goat anti-HPRT serum. Each reaction mixture contained in a final volume of 100 μl: a constant amount of precipitable ^{125}I-labeled HPRT (60 ng), 7 μl of rabbit anti-goat γ-globulin serum, variable amounts of goat anti-HPRT serum, and preimmune goat serum to bring the total goat serum to 0.5 μl. (B) Titration of rabbit anti-goat γ-globulin serum. Each reaction mixture contained in a final volume of 100 μl: a constant amount of precipitable ^{125}I-labeled HPRT (60 ng), 0.1 μl of goat anti-HPRT serum, and variable amounts of rabbit anti-goat γ-globulin serum.

RESULTS

Immunological characteristics of ^{125}I-labeled hypoxanthine phosphoribosyltransferase

The immunological titration of labeled HPRT with goat anti-HPRT serum is presented in Fig. 1A. Approximately 80 to 85% of iodinated HPRT was specifically precipitated by goat serum prepared against unlabeled HPRT. The remaining 15–20% radioactivity may represent HPRT that was denatured prior to iodination or had lost antigenicity as a result of the iodination procedure. The iodinated enzyme can be used to titrate goat anti-HPRT serum with rabbit anti-goat γ-globulin serum. As illustrated in Fig. 1B, 1.5 μl of rabbit serum was required to completely precipitate 0.1 μl of goat serum. Similarly, each lot of rabbit anti-goat γ-globulin serum is tested to determine the amount required to completely precipitate 0.1 μl of goat anti-HPRT serum.

Iodinated enzyme is precipitated with anti-HPRT serum as effectively as unlabeled enzyme. As shown in Fig. 2, when increasing amounts of anti-HPRT serum were added to a mixture of ^{125}I-labeled HPRT and unlabeled HPRT, the same percentage of radioactivity and enzyme activity precipitated.

Radioimmunoassay

Radioimmunoassays can be used to quantitate HPRT cross-reacting material. A competition radioimmunoassay for the pure HPRT and for the enzyme in hemolysates is presented in Fig. 3. The amount of anti-HPRT serum is chosen so that all the anti-HPRT antibody and ^{125}I-labeled HPRT are found in the precipitate. With increasing concentrations of unlabeled enzyme, the percent of ^{125}I-labeled HPRT in the precipitate decreases as a result of competition for the available antibody. When 50% of the precipitable ^{125}I-labeled HPRT appears in the precipitate, the amount of added

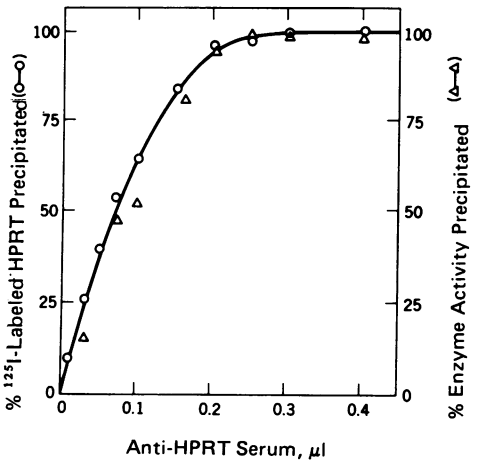

FIG. 2. Coprecipitation of ^{125}I-labeled (O) and unlabeled (Δ) HPRT by goat anti-HPRT serum. Each reaction mixture contained ^{125}I-labeled HPRT (60 ng) and approximately the same amount of purified unlabeled HPRT protein, and variable amounts of goat anti-HPRT serum. The final amount of goat serum (including preimmune serum) was 0.5 μl, and of rabbit anti-goat γ-globulin serum was 7 μl. The enzyme-antibody complex was precipitated as described in *Materials and Methods*. The supernatant was analyzed for enzyme activity and the radioactivity of the precipitate was determined to determine ^{125}I-labeled HPRT precipitated. To correct for nonantigenic ^{125}I radioactivity, the percent ^{125}I-labeled HPRT precipitated was calculated by assigning 100% to the amount precipitated by saturating levels of anti-HPRT serum.

crossreacting material should just equal the amount of ^{125}I-labeled HPRT protein. We use the best fit of a theoretical curve through the data points to determine the concentration of HPRT in a competing sample. The rabbit anti-goat γ-globulin serum ensures complete precipitation of goat antibody-HPRT complexes regardless of the enzyme concentration. The purified and unpurified enzymes competed

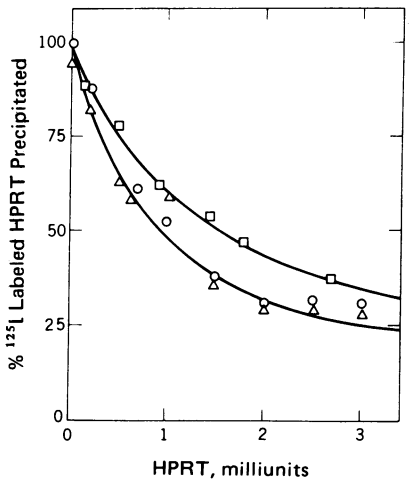

FIG. 3. Radioimmunoassay competition curves. Each reaction mixture contained a constant amount of ^{125}I-labeled HPRT (60 ng), an equivalent amount of goat anti-HPRT serum (0.1 μl), rabbit anti-goat γ-globulin serum, and varying amounts of competing samples. Competing samples are purified HPRT enzyme (9) (10 mU/μg) (O), normal hemolysate enzyme (250 mU/ml) (□), and purified enzyme to which 10 μl of hemolysate from a Lesch-Nyhan patient (J.K.) has been added (Δ). The added Lesch-Nyhan hemolysate had no enzyme activity and no crossreacting material (see Fig. 4).

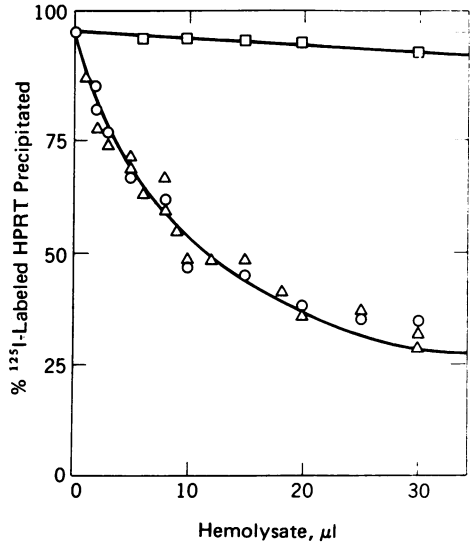

FIG. 4. Radioimmunoassay competition curves for normal human blood (O) and for blood from two different Lesch-Nyhan patients either displaying normal crossreacting material (E.S.) (Δ) or no crossreacting material (J.K.) (□). Each reaction mixture contained a constant amount of ^{125}I-labeled HPRT (60 ng), an equivalent amount of goat anti-HPRT serum (0.1 μl), rabbit anti-goat γ-globulin serum, and varying amounts of competing hemolysates.

almost identically with ^{125}I-labeled HPRT for the antiserum. The presence of hemolysate lacking HPRT crossreacting material did not affect the competition behavior of purified HPRT.

Measurement of crossreacting forms of HPRT in Lesch-Nyhan hemolysates

The radioimmunoassay was used to screen hemolysates from Lesch-Nyhan patients. Two types of results were obtained from the thirty "blind" samples. Six samples had normal levels of crossreacting material. These were two samples of hemolysate from a normal individual and four samples from the Lesch-Nyhan patient (E.S.) with the K_m mutant enzyme. The remaining 24 samples from 15 other Lesch-Nyhan patients contained no detectable HPRT crossreacting material even at high sample volumes (50–90 μl). The radioimmunoassay for hemolysates from a normal individual, for a Lesch-Nyhan patient (J.K.) with no crossreactng material, and from the Lesch-Nyhan patient (E.S.) with the K_m mutant enzyme is shown in Fig. 4.

An experiment to determine the sensitivity of the radioimmunoassay is illustrated in Fig. 5. Different amounts of hemolysate from a normal individual were mixed with hemolysate from a Lesch-Nyhan patient (D.C.) lacking HPRT crossreacting material. The percent of normal HPRT protein was determined from the radioimmunoassay competition curves (Fig. 5A) and is compared to the percent of added normal hemolysate in Fig. 5B. Under these conditions, the radioimmunoassay is clearly able to detect and quantitate as little as 1% of the normal amount of HPRT crossreacting material in hemolysates, or as little as 20 ng of HPRT in a 100-μl radioimmunoassay.

HPRT activity in hemolysates of Lesch-Nyhan patients

The specific activity of HPRT in hemolysates from normal subjects is 150–250 mU/ml of hemolysate (170–220 mg of protein per ml). Twelve of the Lesch-Nyhan hemolysates

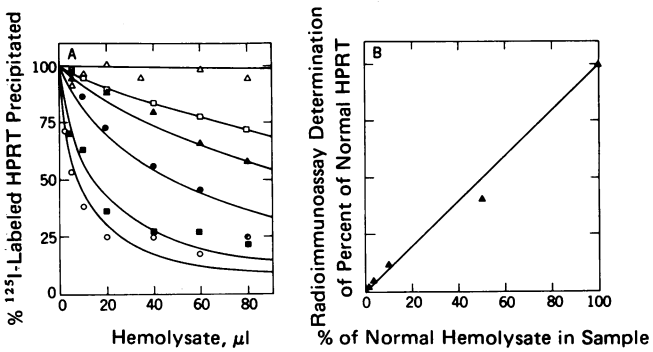

FIG. 5 (A) Sensitivity of radioimmunoassay. Each reaction mixture contained a constant amount of precipitable ^{125}I-labeled HPRT (30 ng), an equivalent amount of goat anti-HPRT serum (0.05 μl), rabbit anti-goat γ-globulin serum, and varying amounts of competing samples. Competing samples are normal human hemolysate (○), Lesch-Nyhan (D.C.) hemolysate (△), and Lesch-Nyhan hemolysate containing 50% (■), 10% (●), 3% (▲), and 1% (□) of normal hemolysate. (B) Comparison of percent of normal HPRT protein in hemolysates determined by radioimmunoassay with percent of normal hemolysate actually present in sample.

(D.G., M.W., D.C., J.F., H.D., V.P., M.G., J.K., J.S., W.E., R.F., and R.G.) contained no detectable HPRT activity (less than 0.1% of normal). Three hemolysates had partial HPRT activity (0.4, 0.2, and 0.1% of normal for B.D., Mike D., and Mark D., respectively). One hemolysate (E.S.) contained 10% of normal HPRT activity when the hypoxanthine and phosphoribosylpyrophosphate concentrations were adjusted from 0.06 mM and 1 mM, respectively, under normal assay conditions (8) to 0.1 mM hypoxanthine and 10 mM phosphoribosylpyrophosphate as previously described (10).

DISCUSSION

The low levels of HPRT activity in Lesch-Nyhan patients could be due either to a defect in the HPRT structural gene or to a defect in mechanisms regulating the expression of the HPRT gene. The K_m mutant enzyme from patient E.S. provides evidence that is some cases the defect is in the structural gene. Immunological techniques can be used to determine if HPRT crossreacting material is present in cases where HPRT enzyme activity cannot be measured. Previous immunological data (3-7), indicating that hemolysates of Lesch-Nyhan patients had normal amounts of HPRT protein, might suggest that all mutations were in HPRT structural gene. However, the radioimmunoassays presented in this paper demonstrate that 15 Lesch-Nyhan hemolysates containing little or no detectable HPRT activity also contain less than 1% HPRT crossreacting material. Thus, the genetic defects in these cases could be at the level of regulation of gene expression.

A defect in the HPRT structural gene could, however, also account for our data. For example, altered forms of HPRT might differ sufficiently in antigenicity so as not to be recognized by antibody to normal HPRT. Another possibility is that altered forms of HPRT might have enhanced lability. *In vivo* studies of mutant HPRT proteins in tissue culture indicate that their stability is often altered relative to that of the normal enzyme (14). Mutant HPRT proteins could be absent in erythrocytes where degraded molecules would not be replaced by newly synthesized proteins. One method to distinguish between these possibilities would be to examine pulse-labeled HPRT protein from fibroblasts of Lesch-Nyhan patients.

This work was supported by U.S. Public Health Service Grant CA-12308 from the National Cancer Institute, Grant DRG-8-F from the Damon Runyon Memorial Fund for Cancer Research, Grant GM-38658 from the National Science Foundation, and Cancer Research funds of the University of California.

1. Lesch, M. & Nyhan W. L. (1964) *Am. J. Med.* **36**, 561-570.
2. Seegmiller J. E., Rosenbloom, F. M. & Kelley, W. N. (1967) *Science* **155**, 1682-1684.
3. Rubin, C. S., Dancis, J., Yip, L. C., Nowinski, R. C. & Balis, M. E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1461-1464.
4. Arnold, W. J., Meade, J. C. & Kelley, W. N. (1972) *J. Clin. Invest.* **51**, 1805-1812.
5. Arnold, W. J. & Kelley, W. N. (1974) in *Purine Metabolism in Man*, eds. Sperling, O., De Vries, A. & Wyngaarden, J. B. (Plenum, New York), pp. 177-186.
6. Muller, M. M. & Stemberger, H. (1974) in *Purine Metabolism in Man*, eds. Sperling, O., De Vries, A. & Wyngaarden, J. B. (Plenum, New York), pp. 187-194.
7. Balis, M. E., Yip, L. C., Yu, T. F., Gutman, A. B., Cox R. & Dancis, J. (1974) in *Purine Metabolism in Man*, eds. Sperling, O., De Vries, A. & Wyngaarden, J. B. (Plenum, New York), pp. 195-202.
8. Olsen, A. S. & Milman, G. (1974) *J. Biol. Chem.* **249**, 4030-4037.
9. Olsen, A. S., & Milman G. (1974) *J. Biol. Chem.* **249**, 4038-4040.
10. McDonald, J. A. & Kelley, W. N. (1971) *Science* **171**, 689-691.
11. Arnold, W. J. & Kelley, W. N. (1971) *J. Biol. Chem.* **246**, 7398-7404.
12. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059-3065
13. David, G. S. (1972) *Biochem. Biophys. Res. Commun.* **48**, 464-471.
14. Capecchi, M. R., Capecchi, N. E., Hughes, S. H. & Wahl, G. M. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4732-4736.