

FOREWORD

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Table of Contents

Front Cover	1
Report Documentation Page	2
Foreward	3
Table of Contents	4
Introduction	5
Body of Report	8
Conclusions	13
References	19

5. Introduction

Tests for mutagenicity, like the Ames test, have been used to screen various chemicals for their ability to cause point mutations and this is often done to try to predict their potential carcinogenicity (1,2). However, such a use of tests for mutagenicity produces many false positive and as well as false negative results in terms of assessing carcinogenic potential and should be supplemented with other types of assays. We are devising tests to detect a type of change in DNA, other than point mutations, that has more recently been shown to frequently be one of the steps in carcinogenesis, namely, chromosomal rearrangements (3). We are using recent molecular biology techniques to develop new methods to screen chemical mixtures for their ability to induce rearrangements of chromosomes.

The prominent role of chromosomal rearrangements, especially translocations and deletions in cancer formation has only lately been widely appreciated (3-5). Chemical as well as "spontaneous" carcinogenesis and generation of birth defects often involve various types of rearrangements of human chromosomes (3-5). These are also a major source of spontaneous abortions. For example, translocations and deletions too small to be visualized by microscopic techniques are important contributors to genetic dysfunction but could not be analyzed until the advent of modern molecular biology methods.

Paternal, as well as maternal, chromosome damage has been implicated in environmentally caused birth defects and infertility (6). Among the agents that have been suspected to cause such damage are Agent Orange, the herbicide 2,4,5-T, and other phenoxy herbicides. Evidence for this comes from laboratory and epidemiological data (7,8). Furthermore, chromosomal rearrangements, birth defects, and cancer are linked together in several groups of genetic diseases in which a high rate of chromosomal rearrangements is coupled with very high rates of cancer as well as disorders of the skin, nervous system, and immune mechanisms (9). This observation is consistent with the major contribution of chromosomal rearrangements to both congenital defects and cancer.

The ability of various chemicals to induce chromosomal rearrangements has

been studied less than their mutagenicity because sensitive assays for these rearrangements are new and the causative role of chromosomal rearrangements in carcinogenesis was firmly established only recently (3-5). Most mutations do not result in changes in protein structure due to the degeneracy of the triplet code, the ability of many amino acids to substitute for one another at a given position in a protein, and the occurrence of many mutations in unimportant regions of chromosomes. In contrast, a much higher percentage of chromosomal rearrangements will be carcinogenic or, if occurring in the male or female germline, will cause birth defects because rearrangements have a high probability of disrupting genes or gene regulatory regions and such disruptions almost always lead to abnormal protein synthesis. Therefore, tests for chromosomal rearrangements could, in many cases, be a better predictor of chemically-caused genetic damage to humans than is general mutagenicity testing.

To test for the ability of suspect chemicals to cause a chromosome translocation that is known to be one of the causes of human cancer, we have used the polymerase chain reaction (PCR). This is an *in vitro* reaction conducted to replicate, up to a million-fold or more, a short DNA region in the presence of a vast excess of other DNA sequences. It involves the use of the DNA sample as a template and two primers that face each other, one each to replicate the top and bottom strands of the DNA sequence of interest in the presence of a vast excess of other DNA sequences. PCR utilizes multiple cycles (usually about 20 - 30) to amplify the DNA. Each cycle involves the following steps: DNA denaturation by heating, annealing of a pair of primers to the DNA template, and replication catalyzed by a thermostable DNA polymerase. The type of PCR assay we have developed is one in which two rounds of PCR are conducted which increases the sensitivity and specificity of the reaction and eliminates interference from the false positive reactions that so often interfere with this technique. When two rounds of PCR are performed, one pair of primers is used for 20 - 45 cycles to obtain a DNA product and then more primer and DNA polymerase are added for 20 - 45 more cycles. With the technique used here, the two rounds are conducted with different pairs of primers. The primers used for the second round are internal

to the pair used for the first round. With this kind of PCR (nested PCR) one DNA fragment is amplified in the first round. Then, a subsequence of that DNA fragment is amplified in the second round. Obtaining the expected sizes of DNA fragments from both rounds is an excellent way to verify that the PCR is amplifying the intended sequence and not some nonspecific sequence.

As little as a single DNA molecule can be detected by PCR followed by blot hybridization (10). Generally when such exquisite sensitivity is achieved, the amount of total DNA present, including nonspecific DNA sequences, is not very high. A great excess of such nonspecific DNA can interfere with the amplification of the targeted DNA sequence and the detection of the specific amplification product. If a very large amount of extraneous DNA is present during such single-molecule detection by PCR, blot hybridization is usually employed to visualize only the desired PCR product (11). We wanted to obtain this same level of sensitivity in the presence of 10^6 genome equivalents of human DNA not containing the exponentially amplifiable sequence of interest using a procedure more amenable to routine analysis than one involving blot hybridization. In this report, we describe such a method involving the use of 22 cycles of amplification with one set of primers for the target sequence (first round of PCR) followed by another 20 cycles (second round of PCR) in which one of the original primers is replaced by a radiolabeled primer complementary to part of the amplified sequence (semi-nested PCR). With this procedure, which should be of use in many types of amplification reactions, we were able to quantitate the number of target molecules in the range of 1 to 15 amplifiable molecules in the original sample.

The targeted DNA in this study is the junction fragment containing the translocated *bcl-2* oncogene region from human chromosome 18 and J_H DNA sequences from the immunoglobulin DNA portion of chromosome 14. This translocation, $t(14;18)$, is observed in a majority of follicular lymphomas (12). There are two highly preferred sites (hotspots) for the *bcl-2*/ J_H translocation (13,14) in the 3' region of the *bcl-2* proto-oncogene and hotspots in the six J_H segments of the immunoglobulin heavy chain locus (15). Because of these hotspots, it is possible to establish PCR assays that can detect a large percentage of these

translocations. In this study we have substituted semi-nested PCR for PCR followed by blot hybridization to amplify and visualize this translocation and we have optimized such amplification reactions and used them for quantitation. Because of the strong clustering of naturally occurring translocation breakpoints on both of the involved chromosomes, the PCR assay that we have developed can detect a large percentage of these translocations.

6. Body

MATERIALS AND METHODS

Materials

PCR was carried out in a thermal cycler (Ericomp) with primers synthesized on an Applied Biosystems synthesizer and purified by electrophoresis on a 20% polyacrylamide gel before use. For the first round of PCR, we synthesized primer 1, 5'-AGAAGTGACATCTTCAGCAAATAAAC-3', from the sense strand upstream of the *bcl-2* gene's major breakpoint region (mbr; a translocation hotspot of ~150 base-pairs [bps] within the 3' untranslated portion of the gene) and primer J_H, 5'-ACCTGAGGAGACGGTGACC-3', a consensus human J_H DNA sequence from the antisense strand of the J_H region of t(14;18) chromosomes. For the second round of PCR, we used primer J_H and radiolabeled primer 3, 5'-ACATTGATGGAATAACTCTGTGG-3', from the sense strand upstream of the *bcl-2* gene's mbr; this sequence is located 67 bps downstream of primer 1. Primer 3 (5 pmol) was labeled with [γ -³²P]ATP (80 μ Ci; 3000 Ci/mmol) in a reaction catalyzed by T4 polynucleotide kinase (20 units) and then mixed with ~300-fold excess of unlabeled primer 3 to a final specific activity of 1000-3000 cpm/pmol. The reactions were catalyzed by Taq polymerase (Promega) or the Stoffel fragment of AmpliTaq DNA polymerase (Perkin-Elmer). The intact Taq polymerase, from batch to batch, gave consistent single-molecule detection in the presence of a vast excess of nonspecific DNA unlike the latter enzyme despite the intact polymerase having some 5'-to-3' exonuclease activity (13). The targeted template for PCR was a t(14;18) *bcl-2*/J_H translocation at the mbr locus in DNA from a human B-lymphoma cell line, SU-DHL-4 (Oncogene Science). The concentration of the commercial SU-DHL-4 DNA stock solution was confirmed by agarose gel electrophoresis of multiple samples against sized-matched DNA

standards upon visualization by fluorescence induced by ethidium bromide (EB). The background DNA that was added to the PCR mixtures to mimic a human DNA sample containing only a few copies of the translocation product in a high background of normal human DNA was normal cerebellum DNA. Contamination of the PCR mixtures was prevented by handling PCR products and reaction ingredients in separate rooms with dedicated pipeting devices and reagents.

Reaction Conditions for the First Round (20-45 Cycles) of PCR

To minimize mis-primed DNA amplification, the Hot Start PCR method (16) employing a paraffin wax (AmpliWax PCR Gem, Perkin-Elmer Cetus) was used in the first round of PCR. The 100 µl-reaction mixtures consisted of different numbers of copies of SU-DHL-4 DNA containing the target translocation; 2.5 units of Taq polymerase (Promega) or 10 units of the Stoffel Fragment of AmpliTaq; dATP, dGTP, dCTP, dTTP (200 µM each); 2.5 mM MgCl₂; 20 pmol each of primers 1 and J_H; 7 µg of normal human brain DNA; and 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 50 mM KCl (for the Promega enzyme) or 12.5 mM Tris-HCl, pH 8.3, 12.5 mM KCl (for the Stoffel fragment). The initial denaturation step was at 95°C for 5 min followed by the indicated number of cycles of 1 min of annealing at 60°C, 1 min of primer extension at 72°C, and 1 min of denaturation at 94°C. In the last cycle, the primer extension time was 10 min. The PCR products were analyzed by electrophoresis of 30 µl of the reaction mixture on a 5% polyacrylamide gel and were visualized by their EB-induced fluorescence.

Reaction Conditions for the Second Round (20 Cycles) of PCR

For the second round of PCR, 5 µl of the undiluted product obtained from the first round of PCR was used in a reaction mixture that was the same as that for the first round except that radiolabeled primer 3 (~2000 cpm/pmol) replaced unlabeled primer 1 and the total reaction volume was 75 µl. Given the much lower sequence complexity of this reaction mixture, to economize, AmpliWax was not used in the second round of PCR. Rather, the mixture was overlaid with two drops of mineral oil and kept on ice until after the 5-µl sample from the first-round PCR product was added through the oil layer to the aqueous layer. The DNA was then subjected to an initial denaturation for 5 min at 95°C followed by 20 cycles of

1 min at 60°C, 1 min at 72°C, and 1 min at 94°C except that the final primer extension was for 10 min at 72°C. The products from the second round of PCR were analyzed by electrophoresis, as above, followed by autoradiography. For quantitative PCR, the specific DNA fragments (~330- and, when observed, 830-bp products) were excised from the gel and their Cerenkov radiation (i.e., light emission in the absence of added scintillants) was measured.

RESULTS

We optimized conditions for PCR detection of a few copies of a DNA containing a chromosome 14/18 translocation at the *bcl-2* proto-oncogene in a background of DNA from approximately 10^6 normal human cells (7 μ g). The source of the translocation-containing DNA was SU-DHL-4 cells in which this t(14;18) rearrangement occurs between the translocation-hotspot *mbr* locus in the 3'-untranslated region of the *bcl-2* proto-oncogene and the fourth of six related J_H regions at the heavy chain immunoglobulin locus. Normal human DNA should give no detectable specific amplification products because the primer-complementary sites in the genomic DNA are on separate chromosomes. After determining that 2.5-5.0 mM Mg^{+2} ions and 60-64°C were optimal for the maximum sensitivity and specificity, we tried to detect only a few copies of the specific translocation product after 40 cycles of PCR with a single pair of primers (primers 1 from chromosome 18 and J_H from chromosome 14). Such primers can amplify a high percentage of naturally occurring, lymphoma-associated translocations (1,3,6,22). We could detect, by EB-induced fluorescence, a specific amplification product of the expected size, ~400 bps, from samples with only two copies of a t(14;18) chromosome in a background of 7 μ g of normal human DNA (data not shown). After 30 cycles of PCR with the same J_H primer and a different *mbr*-specific primer using Southern blotting to visualize the specific products, Negrin et al. (16) obtained a similar specific DNA fragment from as little as one copy of SU-DHL-4 DNA in a background of DNA from 10^5 normal cells. However, when we visualized the PCR products by EB-induced fluorescence, the large amount of background normal human DNA in the samples gave rise to about six bands of nonspecific amplification products.

It was desirable to eliminate the background of nonspecific amplification bands so that the procedure could be used to detect newly arising t(14;18) amplification products whose exact size could not be predicted. We first tried to do this by visualizing amplified DNA by the "oligo extension" assay (17) in which an aliquot of the reaction products after 30-45 cycles of PCR is diluted and subjected to only one cycle of extension of a single primer, namely, the nested, radiolabeled primer 3. The detection limit was ~1 molecule of the t(14;18) chromosome; however, the number and electrophoretic mobility of the specific products was unpredictable. This could be due to the presence of much single-stranded structure in the products after only a single cycle of extension of a nested primer.

The most reproducible, specific, and sensitive method for the detection of only a few molecules of the target DNA in a background of 7 µg of nontarget DNA was semi-nested PCR for two rounds using 22 cycles of amplification with unlabeled primers 1 and J_H in the first round and then 20 cycles with unlabeled primer J_H and labeled primer 3 in the second round (semi-nested PCR; Fig. 1). Fully nested PCR, in which both primers in the second round are changed to match different positions within the amplified region, should have been at least as good but was not possible for general detection of *bcl-2*/J_H translocations because there is only a very short region of high homology between all six J_H gene segments and, therefore, only one ideal consensus J_H primer was available (7). The main specific radiolabeled product obtained from these semi-nested reactions containing as little as one target DNA molecule was a ~330-bp fragment clearly visible after only a single day of autoradiographic exposure (Fig. 1). Sometimes a secondary product of ~830 bps, apparently from priming at the J5 rather than the nearer J4 sequence on SU-DHL-4 DNA, was seen (data not shown) as reported by Negrin et al. (18). No radiolabeled products were ever observed in the samples that contained only the background normal human DNA (Fig. 1 and the last column of Table 1).

There was a very small amount of product electrophoresing as a smear above the major product band when 20-25 cycles were used per round of semi-nested PCR

(Fig. 1) and much more when 30 or more cycles were used for the first round of PCR (data not shown). This smear was largely eliminated when the PCR products were treated with 10 units/ml of S1 nuclease for 8 min at pH 5 (data not shown). When the PCR reaction products were visualized by EB-induced fluorescence, in addition to the specific ~330-bp translocation junction fragment, a major nonspecific product of about ~580 bps was generally observed, even in samples that contained only normal human DNA (data not shown). This amplification product apparently derives from mispriming by just the J_H primer. We observed it in EB-stained gels even when the J_H primer was used with primers for the secondary bcl-2 translocation hotspot, the mcr locus.

Using the above optimal conditions for semi-nested PCR, we determined the relative amounts of radioactivity in the specific radiolabeled PCR products when an average of 1 to 15 target DNA molecules were present in the samples. Each data point shown in Fig. 2 was the average of the results from 3 to 12 independent PCR amplifications. Such replicate determinations were especially important in view of stochastic fluctuation at these extremely low copy numbers. The average amount of specific PCR product was nearly linear in the range of ~1 to 15 target molecules in the starting reaction. The proportionality of the average initial copy number of target molecules to the amount of specific product was further examined by a limiting dilution analysis. We compared the intensity of the ~330-bp band after the second round of PCR when the products from the first round of PCR were subjected to serial dilutions before the second round of PCR amplifications. The results were close to those predicted (Table 2).

By pooling all of our data for semi-nested PCR of samples containing an average of 1 to 15 target molecules of the t(14;18) translocation, we determined whether the probability of detecting the specific radiolabeled product followed the Poisson distribution (Table 1). The close correspondence between the predicted and empirical results further validate this method for detection of a single copy of the target DNA molecule in the presence of a vast excess (7 µg) of background human DNA not containing the specific translocation.

7. CONCLUSIONS

Semi-nested PCR consisting of 22 cycles with two unlabeled primers followed by 20 cycles using an aliquot of the reaction mixture and one unlabeled and one nested labeled primer allowed routine detection of a single translocation-containing molecule of human DNA in the presence of DNA from approximately 10^6 normal human cells. This methodology, which required neither purification of the DNA between the two rounds of PCR nor blot hybridization, also allowed quantitation of very low numbers of the translocation-containing DNA molecules in the presence of a vast excess of background DNA. This type of procedure could be used for routine analyses of chromosomal mutations induced by carcinogens or mixtures of carcinogens used to treat normal human cell lines or cell lines from patients with DNA repair deficiency diseases. It could also be used for detection of very low background levels of chromosomal mutations in disease-free individuals, studies of the persistence of translocation-containing cancer cells in a population of mostly normal cells, or the detection of extremely low numbers of viral or other pathogenic DNA molecules in a high background of normal human cells. Because of the sensitivity and specificity of this methodology, it can be applied to various types of cultured human cells treated with mixtures of military waste products whose carcinogenic potential is being evaluated and the ability of such samples to induce this type of cancer-causing translocation can be determined and compared to their cell toxicity.

Cycles — 22/20 —

— 25/20 —

Copy no. 10 10 4 2 1 0.5 0 10 10 4 2 1 0.5 0



↑



F

1 2 3 4 5 6 7 8 9 10 11 12 13 14

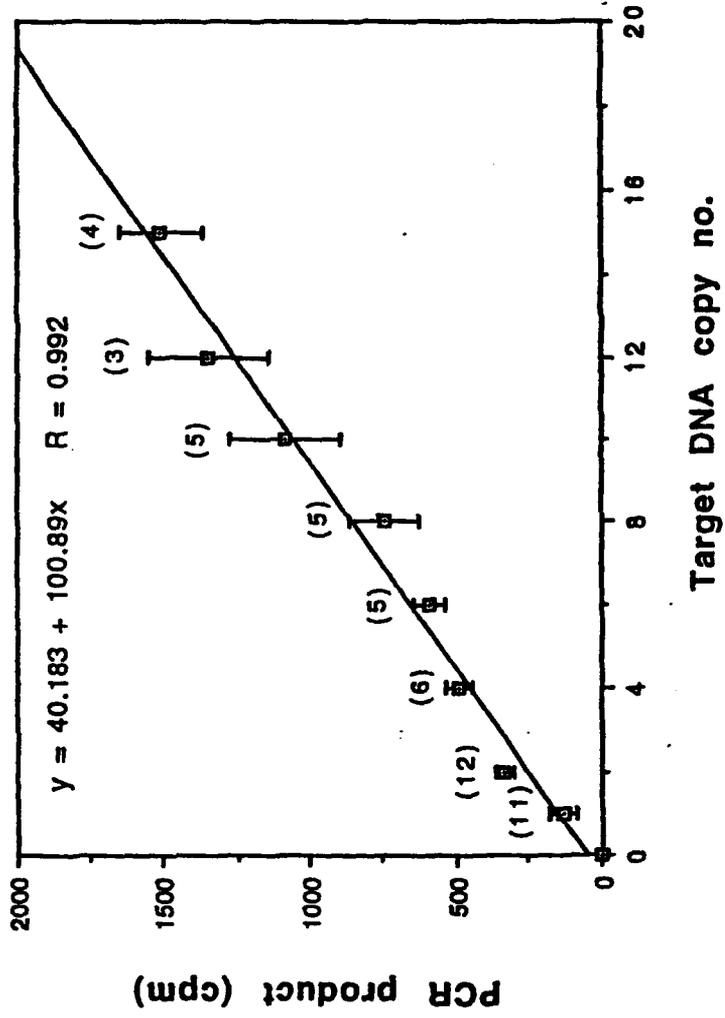


Fig. 1. Optimized detection of the amplification of very low numbers of target molecules in the presence of a vast excess of background DNA by semi-nested PCR. The copy number refers to the expected average number of copies of the *bcl-2/J_H* translocation target molecule that would be present in replicate samples before PCR. Samples containing the indicated copy number of target molecules mixed with 7 µg of normal human brain DNA were subjected to 22 (lanes 1-7) or 25 (lanes 8-14) cycles of amplification with primers 1 and J_H and then a 5-µl aliquot of this first-round PCR product was amplified for 20 cycles in a second round of PCR with primer J_H and ³²P-labeled primer 3. Samples with a copy number of 0.5 will probably have 1 or no target molecules. Gel electrophoresis of the untreated products from the second round of PCR was followed by autoradiography upon 1 day of exposure of the electrophoresis gel to X-ray film. Free ³²P-labeled primer 3 ("F") is indicated.

Fig. 2. Linear relationship between the amount of ³²P-labeled PCR products and average number of *bcl-2/J_H* translocation target molecules. Two rounds of PCR (22 and 20 cycles) were performed on the indicated average number of *bcl-2/J_H* translocation target molecules mixed with 7 µg of normal human DNA using semi-nested primers as in Fig. 1. For each tested average copy number of the target molecules in the initial reaction mixture (including samples with no copies of the translocation product, the "0" data point), 3-12 independent PCR amplifications were performed and the average radioactivity in the specific PCR products (in the ~330-bp band and in the ~830-bp band, when the latter translocation product was also present) is shown. In parentheses above the linear regression curve are the numbers of independent PCR amplifications used for each of the data points. The bar represents the standard error. The correlation coefficient, R, is 0.992.

Table 1. Analysis of the Probability of Obtaining Specific Amplification of Very Low Numbers of the *bcl-2* Translocation-Containing Chromosomes by Semi-Nested PCR

No. of Positive Samples/No. of Tested Samples					
Average Copy No. of the <i>bcl-2</i> Translocation in Tested Samples					
	8	4	2	1	0
Data	10/10	28/29	27/31	26/37	0/30
obtained	1.0	0.97	0.87	0.70	0
Data	0.9997	0.982	0.865	0.632	0
predicted					

"Data obtained" refers to the number of positive samples after semi-nested PCR/the number of tested samples with the given average number of copies of the *bcl-2* translocation. All samples contained a background of 7 µg of normal human DNA (from approximately 10⁶ cells) and were subjected to two rounds of PCR. The products were analyzed by autoradiography. "Data predicted" is from the Poisson distribution used to predict the number of successful amplifications starting with different average low numbers of *bcl-2* translocation-containing molecules per PCR reaction mixture: $1-e^{-n}$, where n is the average number of the *bcl-2* translocation-containing molecules per reaction mixture. According to a chi square goodness-of-fit test, the observed outcomes were not significantly different from those predicted by the Poisson model ($\chi^2 = 0.302$; 4 degrees of freedom; $p = 0.99$).

Table 2. Effect of Varying the Extent of Dilution of the Products from the First Round of PCR on the Yield of Specific Amplification Products from the Second Round of PCR

Copy No. in 1 st PCR	Dilution Factor in 2 nd PCR	Relative cpm Obtained (%)	Predicted Relative cpm (%)
10	1:10	100	100
10	1:100	12.3 12.6	10
10	1:1000	1.2	1
2	1:10	20.9 25.4	20
2	1:100	2.5	2
1	1:10	5.8 8.3	10
1	1:100	0.7	1

Samples which should have an average of 10, 2 or 1 *bcl-2/J_H* translocation target molecules were mixed with 7 µg of normal human brain DNA and amplified for 22 cycles in the first round of PCR. Five microliters of the indicated serial dilutions of this mixture were amplified for 20 cycles of semi-nested PCR and the second-round PCR products analyzed by gel electrophoresis, autoradiography, and quantitation of radioactivity in the specific bands. The radioactivity (cpm) in the specific ~330-bp product (and, where observed, also in the specific secondary 830-bp product) in a given reaction is expressed as a percent relative to that in the reaction shown in the first line of the table. Where indicated, data from duplicate experiments are given. The predicted relative cpm is the expected ratio (expressed as a percent) of the cpm for a given sample relative to that from the reaction shown in the first line of table.

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