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Genome Res. 1993 3: 23-27

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Simultaneous Detection of DNA and RNA by Differential Polymerase Chain Reaction (DIFF-PCR)

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A new technique, the differential polymerase chain reaction (DIFF-PCR), allows the simultaneous amplification of DNA and homologous RNA in a single assay by the combination of DNA–PCR and RNA–PCR on the same target. DNA–PCR amplifies a selected segment of dsDNA, whereas RNA–PCR amplifies a complementary DNA (cDNA), produced by reverse transcription of RNA. In a mixture of target DNA and RNA, DNA is amplified using a combination of sense and antisense primers under high-stringency conditions giving a D-amplicon. RNA is first reverse-transcribed with a primer carrying a nontarget 5′ end into a tagged cDNA at low stringency. Tagged cDNA is subsequently amplified, providing an R-amplicon smaller in size than the D-amplicon. By quantifying the relative amounts of amplified RNA and homologous DNA, a sensitive measure for the transcription rate of a defined DNA segment is obtained. Thus, DIFF-PCR may serve as a useful tool for monitoring gene expression as well as for studying gene regulation and gene function.

For the simultaneous detection of a defined double-stranded DNA (dsDNA) segment and its transcription product RNA in a given sample, a novel technique called differential PCR (DIFF-PCR) was established by combining amplification of DNA (DNA–PCR) and RNA (RNA–PCR). Both DNA–PCR and RNA–PCR are well established as separate methods: In DNA–PCR, which is used for the sensitive detection of dsDNA, a specific DNA segment is amplified by a thermostable polymerase with two oligonucleotide primers that are complementary to known sequences in the target DNA (DNA–PCR).[1] Amplified DNA segments can be analyzed by hybridization of Southern blots using a sequence-specific probe.[2] In RNA–PCR, RNA is first reverse-transcribed with a sequence-specific antisense primer to synthesize a cDNA, from which a segment can be amplified by a DNA polymerase after addition of a sense primer (RNA–PCR).[3,4]

In DIFF-PCR, the techniques of DNA–PCR and RNA–PCR were combined for the detection and differentiation of homologous DNA and RNA segments in the same assay. The principle of DIFF-PCR is presented in Figure 1. Two sequence-specific primers were used to produce a D-amplicon from dsDNA (Fig. 1a). Before starting PCR, reverse transcription (RT) of RNA was performed with an antisense primer, carrying a 5′ end noncomplementary to the target sequence. This partial identity reverse transcription primer (PIRT primer) produced a tagged complementary DNA (PIRT–cDNA) that was amplified after addition of a sense

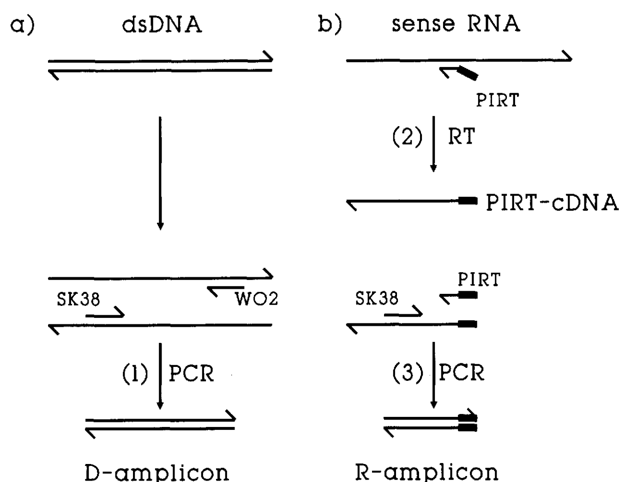

FIGURE 1 Schematic representation of the three reactions involved in DIFF-PCR of a HIV–gag target sequence. (*a*) Production of a D-amplicon (221 bp) in a DNA–PCR (1) with primers SK38 and WO2. (*b*) Detection of RNA by RNA–PCR, composed of RT with a PIRT primer (2) and amplification of the tagged cDNA (3) to an R-amplicon (121 bp). Lines represent HIV nucleic acid strands, with arrows indicating 5′ to 3′ direction. Thick lines represent non-HIV sequence.

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primer to give an R-amplicon smaller in size than the D-amplicon (Fig. 1b). The amplification of dsDNA and of the PIRT–cDNA occurred at the same time with the same sense primer but with different antisense primers. Thus, a set of three primers determined the formation of two amplification products that were specific for the nucleic acid type and were distinguished by gel electrophoresis. Quantification of the reaction products by Southern hybridization was achieved with a probe common to both amplicons. This strategy allowed the simultaneous analysis of DNA and RNA targets of homologous nucleotide sequences in the same sample.

MATERIALS AND METHODS

Setup

1. A 20-μl sample of HIV DNA and RNA was added to a 50-μl mixture containing 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM $MgCl_2$, 400 μM of each dNTP, and 100 pmoles of the RT primer. The reaction mixture was overlaid with liquid paraffin.
2. Denaturation of RNA and primer annealing was performed by heating to 55°C for 10 min, followed by cooling to 37°C.
3. Twenty units of M-MLV RT (GIBCO/BRL, Life Technologies, Inc., Gaithersburg, MD) in 10 μl water was then added. After 30 min of incubation at 37°C, RT was inactivated at 90°C for 10 min.
4. Twenty microliters of water, containing 100 pmoles each of primers SK38 and WO2, 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus), and 20 μg of gelatine was added, and PCR cycling was performed for 30 cycles of 1 min at 99°C, 2 min at 60°C, 3 min at 75°C, with a final 5 min incubation at 75°C.
5. An aliquot was electrophoresed through 2% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME), blotted to a Hybond N+ nylon membrane (Amersham International plc, Buckinghamshire, UK), and hybridized overnight at 50°C with 5′-end-labeled oligonucleotide SK19 as described.[5] Membranes were washed and exposed at −70°C with Fuji RX film using a CAWO intensifying screen.
6. Signal intensities were determined by densitomeric scanning of the autoradiograph on a CD 60 Densitometer (DESAGA, Heidelberg, Germany).

Once a suitable PIRT primer is found, the setup and performance of DIFF-PCR are simple. Ready-to-use mixes (50 μl of RT mix as described in step 1 and DIFF mix containing primers SK38, WO2, and gelatin as described in step 4) can be prepared and stored aliquoted at −20°C for >1 yr without loss of function.

Model

To develop DIFF-PCR, a model system with quantifiable target DNA and target RNA was set up. A segment of the HIV–gag region was amplified by PCR and cloned into a plasmid containing a T7 promoter. Plasmid DNA was quantified photometrically and fluorometrically. Sense HIV RNA and antisense HIV RNA were produced by the T7 RNA polymerase from two clones carrying the insert in opposite orientations. The amount of RNA produced was determined by comparing cRNA and template DNA signal intensities of Northern blots. For amplification by DIFF-PCR, cloned HIV DNA and transcribed cRNA were reacted with the following primers: dsDNA was amplified with primers SK38 and WO2 (Table 1), resulting in a D-amplicon of 221 bp. A PIRT primer was used for the conversion of RNA into PIRT–cDNA, which was amplified to a 121-bp R-amplicon by PCR after adding primer SK38. The three reactions involved in DIFF-PCR were made specific by selectively annealing each primer to its corresponding target only . This was achieved by choosing the appropriate degree of primer/target complementarity, by optimizing the accessibility of the target sequences, and by the different temperatures of the reactions (Table 1).

RESULTS

Uncoupling DNA–PCR from RNA–PCR

Results from DIFF-PCR (Fig. 2) with a mixture of dsDNA and homologous sense RNA showed that amplification of dsDNA to a D-amplicon was independent of RNA–PCR. RNA-specific tagged cDNA was produced in the presence of homologous dsDNA during RT (lanes

TABLE 1 Primer Sequences and their Location in HIV-1

Name	Location in HIV	Matching	RT	R–PCR	D–PCR	Sequence
SK38	1543–1570	28/28	absent	78°C	78°C	5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′
WO2	c1763–c1738	26/26	absent	*	72°C	5′-ATTTTGGACCAACAAGGTTTCTGTCA-3′
SK39	c1657–c1630	28/28	80°C	80°C	80°C	5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′
PIRT14	c1671–c1658	14/28	42°C	86°C	42°C	5′-cgaattcggatccgCTCTAAAGGGTTCC-3′
PIRT11	c1668–c1658	11/28	32°C	84°C	32°C	5′-cgaattcggatccgaagTAAAGGGTTCC-3′
PIRT8	c1665–c1658	8/28	26°C	86°C	26°C	5′-cgaattcggatccatcgatcAGGGTTCC-3′

(GenBank accession number HIVHXB2). Complementary sequences of HIV RNA are denoted by the letter c. Non-HIV bases are shown in lowercase letters. The number of HIV-matching nucleotides for each primer is shown in the third column. The theoretical melting temperatures (T_m) of the primer/target duplexes under standard conditions (hybridization in high salt buffer) were calculated for primer binding to RNA in RT, for primer binding to the R-amplicon in PCR (R–PCR), and primer binding to the D-amplicon (D–PCR) according to the formula [$T_m = 2(A + T) + 4(C + G)$],[6] where A, T, C, and G are the number of nucleotides complementary to the target sequence. These calculated temperatures are not identical to the melting temperatures during DIFF-PCR, but they illustrate the different primer/target stabilities in the three reactions of DIFF-PCR. (*) The absence of the WO2 sequence in the R-amplicon. The probe, used for detection of cloned or amplified HIV target sequences, was SK19 (position 1587–1627, sequence 5′-ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTAC-3′). Oligonucleotides SK38, SK39, and SK19 have been described.[7]

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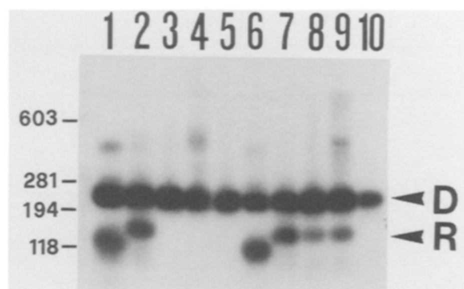

FIGURE 2 Simultaneous detection of HIV DNA and RNA target sequences by DIFF-PCR. HIV plasmid DNA (1 fmole per reaction) was used alone (lanes *1–5*) or together with 25 fmoles of its sense cRNA (lanes *6–10*) as template for DIFF-PCR. RT was performed with primers SK39 (lanes *1,6*), PIRT14 (lanes *2,7*), PIRT11 (lanes *3,8*), PIRT8 (lanes *4,9*) or in the absence of a PIRT primer (lanes *5,10*). DNA was amplified by PCR after addition of primers SK38 and WO2. Amplification products were separated by gel electrophoresis and analyzed by Southern hybridization. The 221-bp D-amplicon, formed by primers SK38 and WO2 is indicated (*D*); the 121-bp R-amplicon, formed by primer SK38 and one of the PIRT primers is also indicated (*R*). The amplification product of primers SK38 and SK39 has a size of 115 bp (lanes *1,6*). Positions of size markers from a ΦX174/*Hae*III digest are indicated at left.

7–9). Although RT does not distinguish between ssDNA and ssRNA,[6] only RNA target produced a PIRT–cDNA in RT. This was made possible by avoiding denaturing of dsDNA. No amplifiable cDNA was produced either without RT, without PIRT primer (lanes 5,10), or from antisense RNA. Amplification of the PIRT–cDNA (RNA–PCR) by primer SK38 and the PIRT primer was not affected by the simultaneously running DNA–PCR with primers SK38 and WO2, because primer WO2 lies upstream of the PIRT location.

Uncoupling RNA–PCR from DNA–PCR

For RNA–PCR, three PIRT primers (28-mers) with different degrees of complementarity to the target RNA were tested for their specificity in directing RT and PIRT–cDNA amplification. They matched the target sequence at their 3′ end with 8 (PIRT8), 11 (PIRT11), and 14 (PIRT14) nucleotides, respectively (Table 1). In addition, a reaction using primer SK39 that has complete complementarity (28 matching bases) and one reaction in the absence of any primer were run in parallel. Amplification of PIRT–cDNA with SK38 and primers PIRT8 or PIRT11, respectively, showed no influence on DNA–PCR, and the production of R-amplicon was specific for sense RNA (Fig. 2, lanes 9,8). Preliminary DIFF-PCR experiments using these primers with RNA isolated from supernatant of HIV-infected cells also led to RNA-specific production of R-amplicons, indicating that the R-amplicon formed was a specific amplification product of the PIRT–cDNA. In contrast, the DIFF-PCR with PIRT14 (lanes 2,7) produced R-amplicons from HIV DNA as well as from a DNA/RNA target mixture without the presence of RT. This non-RNA-specific formation of an R-amplicon was the result of the high complementarity of the PIRT14 primer (14 bases complementary to the target), which enabled annealing to HIV DNA during PCR and, together with primer SK38, its amplification in a DNA–PCR. When primer SK39, which is fully homologous to the HIV target sequence, was used for RT, an untagged cDNA was synthesized. Its amplification by primers SK38 and SK39 was surpassed by the amplification of dsDNA by the same primers, because RNA–PCR was less efficient than DNA–PCR (lanes 1,6).

Quantitation of RNA/DNA levels

Whether the amount of D-amplicon and R-amplicon produced by DIFF-PCR is a function of the amount of DNA and RNA in the sample was determined by amplifying DNA/RNA samples obtained from kinetics of an in vitro T7 RNA polymerase reaction. The effect of increasing sample RNA contents in the DIFF-PCR assay on the ratio of R-amplicon to D-amplicon (R/D ratio) is shown in Figure 3. Using primers PIRT8 and PIRT11, specific amplification products of sense RNA and dsDNA were observed, and the resulting R/D ratio was fairly proportional to the amount of sense RNA in the sample. This allowed an estimation of the RNA quantity in relation to the DNA quantity in a mixture of DNA and homologous RNA. Specificity for amplification of RNA was not retained in DIFF-PCR with primer PIRT14, but the addition of sense RNA resulted in an increased R/D ratio, reflecting the production of a PIRT14-tagged cDNA and its amplification. The nondifferentiating primer SK39, however, showed the same R/D ratio with sense RNA and antisense RNA independent of the RNA concentration.

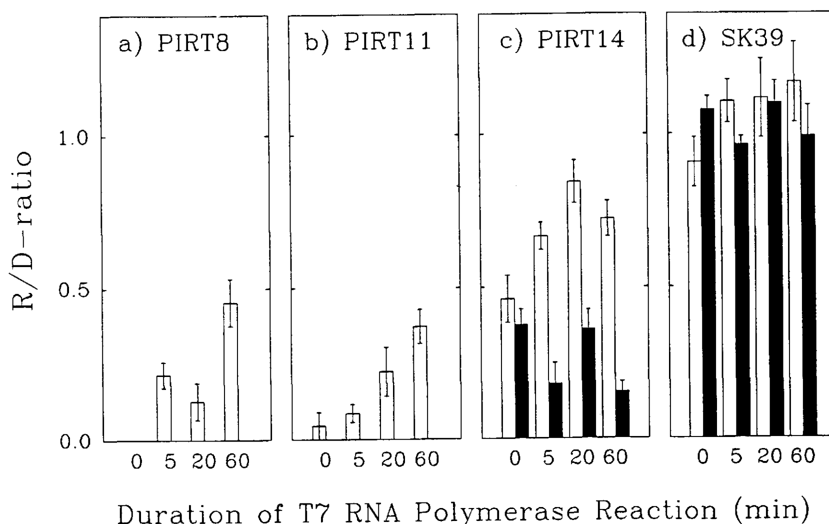

FIGURE 3 Analysis of enzymatical HIV cRNA production by T7 RNA polymerase. Samples taken from T7 RNA polymerase reaction kinetics at 0, 5, 20, and 60 min containing constant amount of target DNA and increasing amounts of RNA were analyzed by DIFF-PCR. Bars represent the ratio of R/D produced by amplification of DNA and sense cRNA (open bars) or DNA and antisense cRNA (solid bars) using RT primers PIRT8 (*a*), PIRT11 (*b*), PIRT14 (*c*), or SK39 (*d*). Using RT primers PIRT8 and PIRT11, respectively, the amplification of RNA was nucleic acid type specific and sequence specific. Autoradiographs from DIFF-PCR were scanned on a CD60 densitometer (Desaga, Heidelberg, Germany). The R/D ratio was calculated by dividing the peak area of the R-amplicon signal by the peak area of the D-amplicon signal. The means ±1 S.E.M. of three independent DIFF-PCR experiments, each with duplicate amplicon analysis, are shown.

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To study the association of the original RNA/DNA ratio in the sample with the R/D ratio after amplification, the RNA and DNA content of samples from the T7 RNA–PCR was determined by Northern hybridization and compared with the R/D ratio obtained by DIFF-PCR (Fig. 4). The R/D ratio did depend on the RNA/DNA ratio of the sample, although the overall efficiency for RNA amplification was 50-fold lower than for DNA amplification. This difference remained constant upon serial dilution of samples (with a RNA/DNA ratio of 1) down to several thousand copies per reaction. This suggests that the lower amplification of RNA is the result of low efficiency of the RT and not preferential amplification of the D-amplicon. The reason for this low efficiency of RT is mispriming of the PIRT primers and not inaccessibility of the RNA, as random-primed RT gives much higher levels of cDNA and more amplification products. The R/D factor is a characteristic of a given set of primers and targets, and its value can be determined empirically. The R/D ratio was determined for the given target/primer combination by assaying samples that contained known amounts of target RNA and DNA. Once this value is determined, the DIFF-PCR is calibrated and simultaneous quantification of RNA in relation to homologous DNA in a sample is possible by DIFF-PCR.

TABLE 2 Requirements for Annealing of Antisense Primers Used in DIFF-PCR

Reaction	Target	Primer WO2	PIRT primer
Reverse transcription	(sense) RNA	no	yes
PCR on cDNA	(sense) ccDNA	no	yes
PCR on dsDNA	sense DNA strand	yes	no

DISCUSSION

Three variables determine the discrimination of DNA and RNA in DIFF-PCR: reaction temperatures, target accessibility, and primer/target complementarity. The differentiating key is the PIRT primer. While the dsDNA remains in double-stranded conformation at low stringency (37°C), the PIRT primer anneals to the RNA and, by elongation with RT, a tag is introduced in the cDNA . Once the tag is introduced, the PIRT primer will distinguish under the high-stringency conditions of PCR annealing (55°C) between native DNA target and the tagged amplification product from RNA. To achieve this discrimination, the PIRT primer must be able to discriminate between the three reactions involved in DIFF-PCR (Fig 1). In each reaction the PIRT primer has to anneal to its specific target, as shown in Table 2, whereas the second antisense primer (WO2) must not anneal. From these requirements the conclusion can be drawn that ccDNA (i.e., the strand complementary to the PIRT–cDNA) must be different from the sense DNA. Because ccDNA is produced from the cDNA during the first cycle of PCR, the tag for later discrimination has to be introduced into the cDNA during RT. This can be performed at low stringency as long as RNA, but not the dsDNA, is accessible to the PIRT primer. This is achieved by heating the sample to 55°C to reduce RNA secondary structure without melting the dsDNA. This denaturing temperature has to be determined empirically for a specific target sequence, as the temperatures for melting of RNA secondary structure and denaturation of dsDNA depend on the GC content and the length of the target sequence.

For the fine tuning of RNA/DNA discrimination the only parameter that can be varied is the number of complementary nucleotides in the PIRT primer, as the temperature settings for the three reactions are limited. We have found that 8 or 11 nucleotides allow discrimination of DNA from RNA, whereas 14 complementary nucleotides lead to incomplete discrimination. In addition, introduction of a sequence tag during RT can also dramatically reduce false positives in RT–PCR.[10]

For quantitative evaluation of DIFF-PCR results, the amplification of the R-amplicon and the D-amplicon have to be equal and the PCR must be in its exponential phase. Similar to competitive PCR with an internal standard, preferential amplification of one of the two targets should be avoided. The equivalence of replication efficiencies is only testable through experiments with known initial target numbers.[11] Unbalanced amplification would lead to a nonlinear relationship between the ratio of PCR products and the ratio of PCR targets.[12] As shown in Figure 4, the R/D ratio is linearly dependent on the DNA/RNA ratio

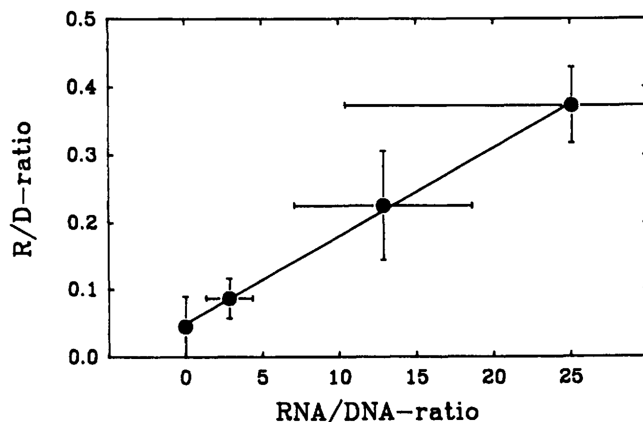

FIGURE 4 Formation of R- and D-amplicons by DIFF-PCR with PIRT11 is proportional to the RNA/DNA ratio of the sample. RNA and DNA amounts in samples from a T7 RNA polymerase reaction kinetics were determined by Northern hybridization and were analyzed further by DIFF-PCR using primer PIRT11. The R/D ratios of DIFF-PCR analysis were proportional to the correspondent RNA/DNA ratios of Northern hybridization analysis. Means ±1 S.E.M. of four Northern hybridizations from three independent DIFF-PCR experiments are shown. For Northern hybridization, 10 μl of T7 RNA polymerase reaction aliquots were subjected to denaturing formaldehyde–agarose gel electrophoresis,[8,9] blotted, and analyzed by hybridization with SK19. RNA/DNA ratios were obtained by dividing the peak area of the runoff transcript signal by the peak area of the plasmid/*Pvu*II digest after densitometric scanning of the autoradiographs. The same samples were diluted 1000-fold and analyzed by DIFF-PCR.

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of the sample in DIFF-PCR. Similar results have been obtained for competitive DNA–PCR[5] and RNA–PCR.[13] In the latter cycles, PCR can reach a plateau as a result of decreased enzyme activity, depletion of reagents, and recombination of reaction products.[14] In latter cycles of DIFF-PCR, the high concentrations of R- and D-amplicons could theoretically lead to "cross recombination" of the two amplicons as a result of partial sequence identity as described for competitive PCR.[11] As long as the ratio of control yield to target yield equals the ratio of control input to target input, the amplification reaction is exponential. Our experimental findings suggest that during DIFF-PCR, amplification is exponential for both targets (Fig. 4).

For application of this method to "true" samples, the method of sample preparation should allow simultaneous isolation of DNA and RNA without changing their relative amounts. The use of glass powder has been described for simple and efficient extraction of either RNA or DNA for PCR.[15] Methods for simultaneous extraction of RNA and DNA have been described,[16–19] but the relative recoveries of RNA and DNA have not been investigated. This remains a problem to be solved for quantitation of RNA/DNA levels by amplification methods.

In contrast to other attempts to discriminate between HIV DNA and RNA,[20] DIFF-PCR does not depend on the presence of a poly(A) tail in the mRNA but allows the sensitive, specific detection of any dsDNA segment together with the corresponding transcription product RNA (e.g., mRNA). Transcription rate is a direct parameter for measuring gene expression, and DIFF-PCR is an attempt to quantitate this parameter by measuring R/D values. We have shown that this method is working in an artificial system using quantified amounts of DNA and RNA. Once adapted to true samples, this novel technique may find applications for the analysis of gene expression on the transcription level and thus may serve as a tool for the study of gene activity.

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Received April 20, 1993; accepted in revised form June 11, 1993.