Amplification of the Total Coding Sequence of the *NF1* Gene from Peripheral Blood Lymphocyte RNA

Ming Hong Shen,¹
Peter S. Harper,
and Meena Upadhyaya

Institute of Medical Genetics, College
of Medicine, University of Wales,
Cardiff CF4 4XN, UK

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with a prevalence of ~1 in 3500. It is characterized by multiple neurofibromas, café-au-lait spots, and Lisch nodules of the iris with a high variability in phenotypic expression.^(1,2) The *NF1* gene was isolated by positional cloning.^(3–5) It is >350 kb in size and encodes a transcript of 11–13 kb. The complete coding sequence is 8457 bp long and contains at least 51 exons.⁽⁶⁾ Analysis of the exon–intron structure of the gene has led to the identification of two alternatively spliced transcripts, GRD II with a 63-bp insertion (exon 23a) and 3'ALT with a 54-bp insertion (exon 48a).^(3,6,7) The search for disease-causing mutations in the gene has proved difficult mainly because of the large size and the high mutation rate of the gene.⁽⁸⁾ Here, we describe a reverse transcriptase–polymerase chain reaction (RT–PCR)-based strategy for amplifying the total coding sequence of the *NF1* gene from peripheral lymphocyte RNA. This amplification should facilitate the further search for mutations in the *NF1* gene.

The strategy involves an initial cDNA synthesis step, followed by two consecutive rounds of PCR amplification. Total RNA was prepared from peripheral blood lymphocytes.⁽⁹⁾ The first-strand cDNA was synthesized by reverse transcription in a 60- μ l reaction containing 300 ng of random hexamers (Pharmacia Biotech), 12 μ l of 5 $\times$ first-strand buffer (GIBCO BRL), 6 μ l of 0.1 M DTT, 15 μ l of 5 mM dNTPs (Pharmacia Biotech), 200 units of Moloney murine leukemia virus (Mo-MLV) reverse transcriptase (GIBCO BRL), 40 units of RNase inhibitor (Boehringer Mannheim), and ~300 ng of total lymphocyte RNA. The reaction was incubated at 42°C for 10 min, 37°C for 60 min, heated at 95°C for 6 min, and cooled in ice for 2 min or stored at –20°C.

Two-round PCR amplifications were performed using the primer sets as listed in Table 1. For the first amplification, each 20- μ l reaction was assembled including 2 μ l of 10 $\times$ PCR buffer (500 mM KCl, 100 mM Tris-HCl, and 20 mM MgCl₂), 200 ng of upper primer, 200 ng of lower primer, 2 units of *Taq* DNA polymerase (Amersham), 10% DMSO or 10% glycerol, and 8 μ l of the first-strand cDNA. In a Perkin-Elmer Cetus thermal cycler, 30 cycles of PCR were programmed at 94°C for 1 min, 55°C for 1

min, and 72°C for 2 min following an initial step of 94°C for 3 min and with a final extension of 72°C for 10 min.

The second amplification was accomplished in a total volume of 50 μ l for each reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.0–2.0 mM MgCl₂, 500 ng of nested upper primer, 500 ng of nested lower primer, 0.2 mM dNTPs, 2.5 units of *Taq* DNA polymerase, 6% DMSO or 10% glycerol, and 1 μ l of the corresponding product of the first amplification reactions (F1 for SF1 and SF2; F2 for SF3; sf3, sf4, and sf5; F3 for SF4, sf6, and sf7; F4 for SF5, sf8, and sf9). PCR was performed as in the first amplification but with different annealing temperatures for different sets of primers as described in Table 1. The amplified products were subjected to electrophoresis on 2% agarose gels.

The second amplification allowed the construction of five or nine overlapping fragments that span an 8694-bp cDNA sequence of the gene (Figs. 1 and 2). The sf5 reaction product was seen as two close bands (Fig. 2). This product is expected to contain the 63-bp insertion region of the GRD II. Because the 63-bp sequence has an *AluI* site, we digested the product with this enzyme to show the presence of the amplified GRD II. The digested product generated an expected band of 450 bp specific for the 63-bp sequence (Fig. 3).

Roberts et al.⁽¹⁰⁾ have used a similar two-round PCR-based strategy to amplify the entire coding sequence of the dystrophin gene from peripheral blood lymphocyte total RNA. Our protocol for the initial reverse transcription step differs from that reported by Roberts et al. in three major points: We utilized random hexamers for the first-strand cDNA synthesis in a single reverse transcription; the primer annealing step at 65°C was also omitted; and we used less quantity of reverse transcriptase, which enabled us to achieve efficient reverse transcription without the inhibitive activity of the excess of reverse transcriptase or its contaminants in the subsequent PCR reaction.⁽¹¹⁾

ACKNOWLEDGMENTS

We are grateful to Dr. N. Thomas for his comments on the manuscript. M.H.S. received a fellowship from the Technical

¹CRC Chromosome Molecular Biology Group, Department of Biochemistry, University of Oxford, Oxford OX1 3QU UK.

Technical Tips

TABLE 1 Primers for Amplification of the *NF1* Gene cDNA by RT-PCR

Reaction ^a	Name	Sequence (5' → 3')	Base position ^b	Exon position	Product (bp)	AT (°C) ^c	MgCl ₂ (mM)
First-round PCR	F1	F1U	CCAGCCGCGCTCTCAATCTCTA	-396--375	1 (5' Utrl)		
		F1L	ATCCTGCTGCACTATCCATAGA	1996-2017	12b-13	2413	55
	F2	F2U	GAGATTAGCTCACAAATG	1714-1731	11-12a		2.0
		F2L	AGTTTTGAACCTCCGTGCAACATA	4759-4782	27b-28	3069 (3132)	55
	F3	F3U	GAATCGAAAGGGGCTTGAAGTTA	4238-4260	24		2.0
		F3L	TGGCTGTAATTTGGTTAGTGCTATTA	6821-6846	37	2609	55
	F4	F4U	CATTGCCTTCCGTTCCAGTTAC	6462-6483	34		2.0
		F4L	CCTCCCACTTTCTTTGCAAGTTGT	8702-8724	49 (3' Utrl)	2263 (2317)	55
Second-round PCR (1)	SF1	SF1U	GGGAGCCTGCACTCCACAGA	-127--108	1 (5' Utrl)		
		SF1L	AATGCCTTTTCCAGGCTATTTATA	636-659	4-5	786	60
	SF2	SF2U	GCCTCTGGGGTTTTATTTTCTCT	412-434	4		2.0
		SF2L	GGAAGTTCTATCTGCCTGCTTATT	1840-1863	12a-12b	1452	60
	SF3	SF3U	CATCTGCAAGAAATTAAGTATCATC	1740-1765	12a		1.5
		SF3L	TTTGGAAGTCCCAGCTTGGTAGA	4715-4737	27b	2998 (3061)	62
	SF4	SF4U	T CAGGAGAAAATTGGGCAGTATCT	4479-4502	26		1.5
		SF4L	T CAGGTCCTTTTAAGCAACTCTC	6763-6785	37	2307	60
	SF5	SF5U	CTCATGGGCAGATAAAGCAGATA	6716-6738	36		1.5
		SF5L	AAAACAGGAAGTGCAGCATTACA	8545-8567	49 (3' Utrl)	1852 (1906)	60
	sf3	sf3U	= SF3U				
		sf3L	CATCATTGTTTCAATGCTAGCTTGC	2952-2976	17	1237	60
	sf4	sf4U	GACTGATACCAATACTCAATTTGTAG	2858-2884	17		1.0
		sf4L	GCCAAGCTGTTGCCCTCGGAAG	3822-3842	22	984	60
	sf5	sf5U	AACAAGGCACAGAATTTGACACA	3563-3585	21	1175 (1238)	60
		sf5L	= SF3L				1.5
Second-round PCR (2)	sf6	sf6U	= SF4U				
		sf6L	TTGTGCTTTGGCATCATCATTAT	5822-5844	31	1366	62
	sf7	sf7U	CTGCCAACAACACCCTCTTTAT	5639-5660	30		1.5
		sf7L	= SF4L			1147	64
	sf8	sf8U	= SF5U				1.5
		sf8L	GCCTTTTAGGAGCCTTTGTGTCT	7584-7606	43	891	60
	sf9	sf9U	CAACCTTCTCAGGCCAACACTA	7519-7540	42		1.5
		sf9L	= SF5L			1049 (1103)	60

^aThe products of reactions F2, SF3, and sf5 contain the alternatively spliced 63-bp insertion, exon 23a; the products of reactions F4, SF5, and sf9 contain the alternatively spliced 54-bp insertion, exon 48a.

^bThe *NF1* cDNA sequence was numbered according to the NF1 Genetic Analysis Consortium.

^c(AT) Annealing temperature.

FIGURE 1 Amplification of the *NF1* coding sequence with five overlapping nested primer sets (lane 1) λ DNA (*Hind*III-*Eco*RI) marker; (lanes 2-6) the products of reactions SF1, SF2, SF3, SF4, and SF5; (lane 7) 1-kb ladder.

FIGURE 2 Amplification of the *NF1* total coding sequence with nine overlapping nested primer sets. (Lanes 1-9) The products of reactions SF1, SF2, sf3, sf4, sf5, sf6, sf7, sf8, and sf9; (lane 10) 1-kb ladder.

FIGURE 3 Restriction analysis of the alternate transcript GRD II with *Alu*I. (Lane 1) One-kilobase ladder; (lane 2) the digested products of reaction sf5.

Cooperation of the Chinese Education Committee and the British Council. This work was supported by Action Research.

REFERENCES

1. Riccardi, V.M. and J.E. Eichner. 1986. *Neurofibromatosis: Phenotype, natural history and pathogenesis*. Johns Hopkins University Press, Baltimore, MD.
2. Huson, S.M., D.A. Compston, P. Clark, and P.S. Harper. 1989. A genetic study of von Recklinghausen neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of parental transmission on severity. *J. Med. Genet.* **26**: 704–711.
3. Cawthon, R.W., R. Weiss, G. Xu, D. Viskochil, M. Culver, J. Stevens, M. Robertson, D. Dunn, R. Gesteland, P. O'Connell, and R. White. 1990. A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* **62**: 193–201.
4. Viskochil, D., A.M. Buchberg, G. Xu, R.M. Cawthon, J. Stevens, R.K. Wolff, M. Culver, J.C. Carey, N.G. Copeland, N.A. Jenkins, R. White, and P. O'Connell. 1990. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* **62**: 187–192.
5. Wallace, M.R., D.A. Marchuk, L.B. Andersen, R. Letcher, H.M. Odeh, A.M. Saulino, J.W. Fountain, A. Brereton, J. Nicholson, A. Mitchell, B.H. Brownstein, and F.S. Collins. 1990. Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. *Science* **249**: 181–186.
6. Viskochil, D., R. White, and R. Cawthon. 1993. The neurofibromatosis type 1 gene. *Annu. Rev. Neurosci.* **16**: 183–205.
7. Gutmann, D.H., L.B. Andersen, J.L. Cole, M. Swaroop, and F.S. Collins. 1993. An alternatively-spliced mRNA in the carboxy terminus of the neurofibromatosis type 1 (NF1) gene is expressed in muscle. *Hum. Mol. Genet.* **2**: 989–992.
8. Upadhyaya, M., D.J. Shaw, and P.S. Harper. 1994. Molecular basis of neurofibromatosis type 1 (NF1): Mutation analysis and polymorphisms in the NF1 gene. *Hum. Mut.* **4**: 83–101.
9. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
10. Roberts, R.G., T.F.M. Barby, E. Manners, M. Bobrow, and D.R. Bentley. 1991. Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes. *Am. J. Hum. Genet.* **49**: 298–310.
11. Fehlmann, C., R. Krapf, and M. Solioz. 1993. Reverse transcriptase can block polymerase chain reaction. *Clin. Chem.* **39**: 368–369.

Received October 11, 1994; accepted in revised form February 1, 1995.

Amplification of the total coding sequence of the NF1 gene from peripheral blood lymphocyte RNA.

M H Shen, P S Harper and M Upadhyaya

Genome Res. 1995 4: 311-313

References

This article cites 10 articles, 3 of which can be accessed free at:
<http://genome.cshlp.org/content/4/5/311.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

Focused on your science.

USC
SCIENTIFIC
SIDE BY SCIENCE

To subscribe to *Genome Research* go to:
<https://genome.cshlp.org/subscriptions>