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SSCP variants are identified by comparing the migration of corresponding PCR fragments generated from a normal sample and run in the same gel. An ideal scheme for the most efficient mutation analysis of cDNA is diagrammed in Figure 1. Only two multiplex reactions are required to cover a target sequence of 892-bp cDNA. In this example, each multiplex reaction uses four PCR primers (1F, 1R, and 3F, 3R or 2F, 2R, and 4F, 4R) that generate two non-overlapping 250-bp fragments separated by 178 bp and a 678-bp fragment that is the amplification product produced from the proximal forward primer (1F or 2F) and the distal primer (3R or 4R). Together, the 250-bp fragments from both multiplex reactions cover the entire 892-bp target sequences with 36-bp overlaps between the PCR fragments in the independent multiplex reactions. An overlap of at least 36 bp is recommended for 18-bp primers to ensure that point mutations in the primer annealing sequence of one fragment will be detected in the fragment from the other reaction. The 678-bp fragment includes the individual 250-bp fragments and the intervening 178-bp sequence. While the PCR primers, fragment sizes and the number of multiplex reactions will vary according to the gene of interest, this strategy is a rapid and efficient method for mutation screens of a large number of samples.

Following the generalized scheme outlined in Figure 1, we have screened for mutations in patients with X-linked Adrenoleukodystrophy (X-ALD) (4). In this instance, four multiplex reactions were carried out to screen for mutations in the X-ALD cDNA (2376 bp). These multiplex reactions generated non-overlapping short PCR fragments ranging from 239–265 bp and longer fragments ranging from 648–882 bp that contain two of the short fragments and the intervening sequences between them. Figure 2A demonstrates a mutation in the X-ALD gene detected by SSCP analysis of a multiplex reaction analyzed without prior separation of fragments (5697A lane, altered bands in boxes). Sequence analyses of the corresponding larger fragment (Figure 2B) isolated from an agarose gel confirmed the SSCP variation and allowed identification of the P543L missense mutation.

A two-step multiplex PCR-SSCP strategy, followed by sequence analysis of a separate PCR fragment generated from the previously identified SSCP variant, has been used to screen for mutations in several different genes (1,3,6). These methods require at least two PCR amplifications. The first one is a multiplex reaction that generates multiple fragments of similar sizes for the SSCP analysis. The second PCR generates the fragment for sequencing analysis and confirmation of the mutation detected by SSCP.

The method described here is a one-step strategy that integrates these two reactions into a single step without compromising the confirmation role of the PCR fragment used for sequencing. The co-amplification of the smaller multiplex fragments for PCR analysis and a larger fragment suitable for sequencing in one reaction tube facilitates the analysis of a large number of samples, especially if the source of DNA is limited. Once optimized, this methodology allows efficient and reliable “serial screening” of a large number of samples with no need to subsequently amplify specific PCR fragments for each SSCP variation that was previously found. Using this strategy, we were able to rapidly identify 32 mutations in the X-ALD gene.

REFERENCES

1. Castresana, J.S., L. Gómez, P. Garua-Miguel, A. Queizan and A. Pestana. 1997. Mutational analysis of the p16 gene in human neuroblastoma. *Mol. Carcinog.* 18:129-133.
2. Fan, E., D.B. Levin, B.M. Glickman and D.M. Logan. 1993. Limitations in the use of SSCP analysis. *Mutat. Res.* 288:85-92.
3. Kneppers, A.L.J., P.P. Deutz-Terlouw, J.T. Dunnen, G.J.B. van Ommen and E. Bakker. 1995. Point mutations screening for 16 exons of the dystrophin gene by multiplex single-strand conformation polymorphism analysis. *Hum. Mutat.* 5:235-242.
4. Lachtermacher, M.B.R., H.N. Seuanez, A.B. Moser, H.W. Moser and K.D. Smith. 2000. Determination of 30 X-linked Adrenoleukodystrophy mutations, including 15 not previously described. *Hum. Mutat.* 15:348-353.
5. Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi and T. Sekiya. 1989. Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* 86:2766-2770.
6. Phillips, K.A., K. Nichol, H. Ozcelik, J.

Knight, S.J. Done, P.J. Goodwin and I.L. Andrusis. 1999. Frequency of p53 mutations in breast carcinomas from Ashkenazi Jewish carriers of BRCA1 mutations. *J. Natl. Cancer Inst.* 91:469-473.

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RNA Amplification Technique, NASBA, Also Amplifies Homologous Plasmid DNA in Non-Denaturing Conditions

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NASBA is described as a highly sensitive amplification method specific for RNA. It permits the detection of eukaryotic gene transcription even if the RNA samples contain traces of genomic DNA (2). Recently, NASBA was de-

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scribed as being able to specifically amplify cellular human interferon- β mRNA and not the corresponding intronless gene (4). Thus, NASBA may be a useful tool to determine the possible association between human endogenous retrovirus (HERV) RNA expression and some pathologies (6). We have investigated whether NASBA is also an appropriate method for the specific detection of HERV-W RNA expression in multiple sclerosis patient cells (1,3). To do that, we defined the optimal NASBA amplification conditions using in vitro-transcribed HERV-W *pol* RNA from plasmid DNA.

A 620-bp fragment of the HERV-W *pol* region (nucleotides 602–1222, GenBank® accession no. AF009668) was cloned into *Pst*I and *Xba*I pSP64 PolyA plasmid (Novagen, Madison, WI, USA), which possesses only the SP6 RNA polymerase promoter. pSP64-*pol* plasmids were purified using phenol/chloroform extraction, RNase A treatment and further phenol/chloroform extraction. *Eco*RI-linearized pSP64-*pol* plasmids were transcribed using the MAXIScript™ kit (Ambion, Austin, TX, USA), and the RNA obtained were digested with RNase-free DNase I and purified with the RNeasy® kit (Qiagen, Valencia, CA, USA). The *pol* transcripts and pSP64-*pol* plasmids were quantified by photometry at 260 and 280 nm, and NASBA was performed (5). Briefly, HERV-W *pol* RNA or pSP64-*pol* plasmid solutions were added to a reaction mixture containing 15 mM MgCl₂, 90 mM KCl, *pol*36 and *pol*40 primers (nucleotides 777–805 and 1005–1029, GenBank accession no. AF009668). A 2.5- μ L aliquot of the 200-nucleotide NASBA products was analyzed using a specific detection system, the Enzyme-Linked OligoSorbant Assay (ELOS) (1,7). The ELOS absorbance cutoff, defined as the mean of the water controls of the NASBA plus three SD, was 0.05 at 492 nm (A_{492}).

Here, we describe a HERV-W *pol* NASBA system coupled to a colorimetric detection method (ELOS) that permitted the detection of 10 molecules *pol* RNA/assay (Figure 1A). However, the HERV-W *pol* NASBA system presented a positive amplification using more than 10⁴ molecules pSP64-*pol*

plasmid DNA/assay (Figure 1B). Our results indicate that the NASBA technique cannot amplify RNA exclusively because an important quantity of homologous plasmid DNA that carries sequences corresponding to the primers used could produce a positive signal (Figure 1B). Note that plasmid DNA was not denatured during the 65°C heating step because the same sensitivity was obtained when this step was omitted. Our results demonstrated that plasmid DNA could be amplified in a NASBA system and showed that plasmid DNA could not be used as a nega-

tive control in NASBA experiments.

From these results, we concluded that NASBA is a highly sensitive technique for the detection of infectious agents (5) and the expression of single-copy genes (4). But homologous double-stranded plasmid DNA can be amplified if it is present in too high a copy number. Recently, it was shown that NASBA does not amplify an intronless gene from 100 ng eukaryotic genomic DNA (about 28 000 copies) and that NASBA was highly sensitive for transcripts of the gene (4). These results do not conflict with the amplification of

Figure 1. Detection of NASBA amplification products with a specific colorimetric detection method. After NASBA amplification, products were detected by ELOS at 492 nm (A_{492}). (A) HERV-W *pol* RNA NASBA amplification. Decreasing amounts of synthetic HERV-W *pol* RNA, ranging from 0–1000 copies, were subjected to NASBA amplification in the optimal conditions used for the primers. (B) pSP64-*pol*-linearized plasmid NASBA amplification. Decreasing amounts of pSP64-*pol* plasmids ranging from 0–10⁸ copies were subjected to NASBA amplification under the same conditions used for RNA amplification.

10 000 copies of plasmid DNA described in this paper. Indeed, the accessibility of the target is higher on plasmid DNA than on genomic DNA that has a more complex conformational organization. In addition, replicate intermediates of the plasmid are single-stranded and so are a template for NASBA amplification. Therefore, NASBA is not a suitable method to detect transcription of plasmid DNA.

From these results, we determined that for the highly sensitive detection of eukaryotic gene transcription with NASBA, massive contamination of the RNA sample with genomic DNA may be a problem if the template is multiple copy-number genes (e.g., HERV) and if the sample is derived from a tissue with high mitotic activity. In general, DNA molecules are not static replication and transcription events that lead to the formation of single-stranded regions or permit specific primer hybridization or amplification by NASBA.

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REFERENCES

1. Blond, J.L., F. Besème, L. Duret, O. Bouton, F. Bedin, H. Perron, B. Mandrand and F. Mallet. 1999. Molecular characterization and placental expression of HERV-W, a new human endogenous retrovirus family. *J. Virol.* 73:1175-1185.
2. Compton, J. 1991. Nucleic acid sequence-based amplification. *Nature* 350:91-92.
3. Garson, J.A., P.W. Tuke, P. Giraud, G. Paranhos-Baccalà and H. Perron. 1998. Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. *Lancet* 351:33.
4. Heim, A., I.M. Grumbach, S. Zeuke and B. Top. 1998. Highly sensitive detection of gene expression of an intronless gene: amplification of mRNA, but not genomic DNA by nucleic acid sequence based amplification (NASBA). *Nucleic Acids Res.* 26:2250-2251.
5. Kievits, T., B. Van Gemen, D. van Strijp, R. Schukkink, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan and P. Lens. 1991. NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Virol. Methods* 35:273-286.
6. Löwer, R., J. Löwer and R. Kurth. 1996. The viruses in all of us: characteristics and biological significance of human endogenous retroviral sequences. *Proc. Natl. Acad. Sci. USA* 93:5177-5184.
7. Perron, H., J. A. Garson, F. Bedin, F. Besème, G. Paranhos-Baccalà, F. Komurian-Pradel, F. Mallet, P. W. Tuke et al. 1997. Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 94:7583-7588.

New Genetic Tools for Use in the *Rhizobiaceae* and Other Bacteria

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During the past decade there has been considerable interest in the isolation and characterization of microbes from diverse environments. Unfortunately, genetic experiments in prokaryotes other than *E. coli* often are limited by the availability of stable, compatible vectors with various drug resistance markers. For example, we found that in our organism of interest—the symbiotic soil bacterium *Sinorhizobium meliloti* strain 1021 (derivative of SU47)—plasmids of the incompatibility groups IncQ and IncW are not stably maintained under some culture conditions (7,12,16).

Previously, our group reported the characterization of an autonomously replicating sequence (ARS) from the *S. meliloti* pSymB replicon (14). Although we constructed vectors using this ARS, we found that they were unstable in the absence of antibiotic selection (M. Barnett, unpublished results). Recently, we have made extensive use of derivatives of the broad-host-range plasmid pBBR1 from *Bordetella bronchiseptica*, which we describe here. With the use of new plasmid replicons, we needed additional resistance markers to use multiple vectors effectively, especially in the presence of marked genomic mutations. Here, we report the construction of a new Tn5 derivative carrying hygromycin resistance (Hy^r); construction of two new pBBR1MCS derivatives containing spectinomycin resistance (Sp^r) or Hy^r; and construction of pBluescript®-derived (Stratagene, La Jolla, CA, USA) suicide vectors, carrying neomycin resistance (Nm^r), Sp^r or Hy^r.

The most commonly used resistance markers in *S. meliloti* are gentamicin, neomycin, spectinomycin, streptomycin and tetracycline. Since other antibiotics such as chloramphenicol (Cm) and β -lactam compounds are not usable in our strain, we sought candidates for an additional resistance marker. Hygromycin resistance has not previously been exploited in the *Rhizobiaceae* but had