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MS for Identification of Single Nucleotide Polymorphisms and MS/MS for Discrimination of Isomeric PCR Products

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ESI (electrospray ionization) MS and tandem mass spectrometry (MS/MS) were used for the analysis of single nucleotide polymorphisms (SNPs) and more complex genetic variations. Double-stranded (ds) PCR products were studied. PCR products of the proline [5′-*x*(G_{17})-*x*(C_{38})*x*-3′] and arginine variants [(5′-*x*(G_{17})-*x*(G_{38})*x*-3′] of the p53 gene are distinguished by an SNP (cytosine or guanine) and were discriminated using both quadrupole and quadrupole ion trap MS analysis. A 69 bp arginine mutant PCR product [5′-*x*(C_{17})-*x*(G_{38})*x*-3′] with a negating switch has the same mass as the proline variant but was readily distinguishable on ion trap MS/MS analysis; fragments containing the mutation site, but not the polymorphism, were identified. The 69 bp PCR products were restriction-enzyme-digested, to create 43 bp fragments. ESI quadrupole ion trap MS/MS analysis of the 43 bp product-ion spectra readily demonstrated both polymorphism and negating switch sites. MS and MS/MS are powerful and complementary techniques for analysis of DNA. MS can readily distinguish SNPs but MS/MS is required to differentiate isomeric PCR products (same nucleotide composition but different sequence).

In a population, one finds a single nucleotide polymorphism (SNP) every 200−300 bases.[1] Other sequence variations include copy number changes, insertions, deletions, duplications, and rearrangements. Sequencing of an entire human genome will be accomplished in the next few years.[2] Another challenge will be studies of genetic diversity within the population; recognition of SNPs will be sufficient in most cases. However, ideally, any large-scale studies of genetic diversity should detect other more-extensive genetic changes.

Gel electrophoresis is commonly employed for the determination of molecular weight (MW) of polymerase chain reaction (PCR) products by relative electrophoretic mobility. To identify SNPs, the use of high-resolution electrophoresis (e.g., single-strand conformation polymorphism [SCCP]) and Sanger sequencing is required. SSCP can distinguish many PCR products, but it does not provide structural information. It merely demonstrates that a change in mobility has occurred. Also, certain PCR products of differing sequence can migrate similarly on SSCP analysis.[3,4] Alternatively, Sanger sequencing is currently too expensive for large-scale population studies. The Human Genome project recognizes that new technology needs to be developed which can dramatically improve the throughput and decrease the cost of analysis. Hybridization using chip-based technology is one of the leading contenders for population studies. Unfortunately, hybridization does not require that two DNA sequences be identical for binding to occur. Thus, even with optimization of binding conditions, there will always be false positives.[5]

MS approaches for genomic characterization may be divided into two types. In the first approach, MS has been used to analyze Sanger sequencing ladders. MALDI-TOF MS has been most commonly used for analyzing complex sequencing reaction

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mixtures, since it produces a single mass peak with a charge of −1 for each oligonucleotide component. MS can potentially replace conventional gel electrophoresis sequencing ladders.[6,7] The second MS approach assesses mass changes between two pieces of DNA.[8−13] For example, previously uncharacterized PCR products are compared with a PCR product of known sequence, identifying insertions/deletions as well as SNPs. Insertions/deletions require discrimination of losses or gains equal to the mass of a nucleotide (289→329 Da). Nucleotide substitutions (including transitions and transversions) range from 9→40 Da. For example, a polymorphism in the human p53 gene can be a G or C (G→C),[14] which would be recognized by a 40 mass unit change.[13] However, as the size of the nucleic acid increases, both sensitivity and mass accuracy are adversely affected.[12,13,15−19]

Using MALDI-TOF MS, up to 69 mers differing by an SNP have been successfully discriminated.[12] ESI with high-resolution MS (magnetic sector or FTICR) analysis also allows differentiation of SNPs.[8,10] Because of the lower sensitivity of magnetic-sector instrumentation, the PCR product is generally cut with a restriction enzyme into smaller pieces of DNA containing the SNP site.[8,9] Only with ESI FTICR MS analysis have SNPs been detected in intact PCR products above 100 bp.[10,11] Recently, ESI quadrupole MS analysis has been shown to have the mass accuracy to detect SNPs in intact PCR products, up to 62 mers.[13] Commonly in MALDI-TOF analysis, the sequence of the DNA is generally known, allowing the design of the primer extension reactions or probes to detect the presence of known SNPs.[20,21]

ESI MS and MS/MS has potential for evaluating larger PCR products for known (or unknown) SNPs or other genetic changes relative to human genomic PCR products of known sequence. In MS/MS, intact DNA is broken into fragments. For short oligonucleotides (e.g., 15 mers), sequence can be inferred from product ion spectra a priori.[22] Oligonucleotides up to 100 bases have also been sequenced without resorting to sequencing ladders.[23,24] Nozzle-skimmer fragmentation (producing dinucleotides) followed by MS/MS analysis also allows determination of adjacent nucleotides in larger nucleic acids.[25] However, complete sequencing from fragmentation patterns of large pieces of DNA remains challenging at the present time. MS/MS also has potential as a fingerprinting tool to recognize sequence variations. Isomeric oligonucleotides, of the same mass, have been discriminated on quadrupole MS/MS analysis.[26] Ion trap MS/MS has also been used to differentiate short fragments of the sense and antisense strands of the APC gene, differing in sequence but having the same mass/nucleotide composition.[27] The current research demonstrates the use of MS for differentiation of intact PCR products of different mass (SNPs) and MS/MS fingerprinting for discrimination of PCR products with the same mass/nucleotide composition but different sequences.

EXPERIMENTAL SECTION

Materials. Plasmids containing cDNA of the human p53 gene and ampicillin resistance were provided by Dr. D. Reisman, Department Biol. Sci., USC. Plasmid pC53-SN encodes a proline ("pro", CCC) at codon 72 in exon 4 of the p53 gene and plasmid p53H13 encodes an arginine ("arg", CGC). Plasmids were transformed into *Escherichi coli* DH5α cells. DNA concentration was determined using absorbance at 260 nm.

DNA Preparation. Transformed *E.coli* DH5α cells were cultured at 37 °C on LB agar containing ampicillin. Plasmid DNA was purified using Strataprep Plasmid Miniprep Kit (Stratagene, La Jolla, CA). One and one-half milliliters of bacterial culture were pelleted and resuspended in 100 μL of buffer solution (50 μg/mL ribonuclease). One hundred microliters of lysis solution (0.2 M NaOH, 1% weight/volume sodium dodecyl sulfate) and a 125-μL DNA binding solution were added. After centrifugation, the plasmid DNA in the supernatant was bound to a matrix which was washed and then eluted with 100 μL of water.

Polymerase Chain Reaction. For PCR of p53 gene products amplified from plasmid DNA, 100-μL reactions contained: 10 μL of *Pfu* buffer [200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1.0% Triton X-100, and 1 mg/mL BSA] (Promega, Madison, WI), 8 μL of a mixture containing 10 mM each of dNTP (dCTP, dTTP, dATP, and dGTP), 1.0 μL of cloned *Pfu* polymerase (2.5−3.0 U/μL) (Promega Corporation, Madison, WI), and 56 μL of water (Fluka Chemika-Biochemika, Ronkonkoma, NY). All reagents were treated with 5 μL of CalBiochem deoxyribonuclease I (2 U/uL)(Behring Diagnostics, La Jolla, CA) for 30 min at 37 °C. The deoxyribonuclease was then inactivated by incubation for 5 min at 95 °C prior to the addition of 8 μL of each primer (10 pm/μL) and ∼75 ng of plasmid DNA. Primers used for the amplification of the 69 bp PCR products containing the pro and arg p53 variants were p53+69 (5′-CCAGATGAAGCTCCC-3′) (forward) and p53−69 (5′-CGGTGTAGGAGTCGCTGGTG-

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3′) (backward). The primer for the 69 bp products containing a site-directed mutation (arginine-negating switch [arg-NS]) was p53+69−NS (5′-CCAGATGAAGCTCCCACAATGCC-3′) (forward). The other primer was unchanged. PCR was performed using an Idaho Technologies Rapidcycler (Idaho Falls, ID). Cycling parameters were optimized for thin-walled microcentrifuge tubes containing 100-μL reaction mixtures. Products were generated using a 5-min denaturing step at 95 °C, followed by 40 cycles of 1 min of denaturing at 95 °C, 1 min of annealing at 53 °C (for primer pair p53+69 and p53−69) or 55°C (for primer pair p53+69-NS and p53−69), and elongation for 1 min at 72 °C. The final step was a 5-min elongation at 72 °C.

Purification of PCR Products and Restriction Digests. Two 100-μL PCR reactions were used. Precipitations were carried out using a final concentration of 2 M ammonium acetate (NH_4OAc) and 2.5 volumes of ice-cold 100% ethanol. After overnight incubation at −20 °C, solutions were centrifuged for 30 min at 14 000 rpm at room temp. The pellet was washed once with 200 μL of 100% ethanol and once with 200 μL of 70% ethanol by vortexing followed by centrifugation. The washed pellet was resuspended in 22 μL of sterile water and stored at 4 °C.[8,13,28−30] PCR products, prior to and after purification, and also after restriction enzyme digestion (see below), were separated on 3% agarose gels (Agarose III, Amresco, Solon, OH) containing ethidium bromide and then examined under UV light. For analysis of PCR product using the quadrupole ion trap mass spectrometer, ethanol precipitated PCR products were further desalted using microdialysis. A 200-μm i. d. dialysis tubing with a 13 000 MW cutoff (Spectrum, Houston, TX) was used as previously described.[31] Briefly, after the dried sample was resuspended in 1 mM NH_4OAc it was infused into the fiber at 2.5 μL/min. The 10 mM NH_4OAc dialysis buffer was delivered in a countercurrent direction at 300 μL/min outside of the fiber.

69 bp PCR products were cut into 43 bp and 26 bp products using Hae III, which cuts double-stranded (ds) DNA at GG∧CC. Twenty-microliter reactions were used which included 5.4 μL of water, 2 μL of 10× bovine serum albumin, 2 μL of 10× buffer C (100 mM HCl [pH 7.9], 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT) (Promega Corporation, Madison, WI), 10 μL of precipitated DNA, and 0.6 μL of Hae III (10 U/mL) (Promega Corporation, Madison, WI). Reactions were incubated at 37 °C for 3 h. Four 20-μL reactions were pooled for ethanol precipitation as described above.

ESI MS and MS/MS Analysis. Analysis was performed using a Quattro 1 quadrupole mass spectrometer (Micromass, Danvers, MA). Ten microliters of precipitated PCR products were analyzed with the addition of 10 μL of 100% acetonitrile and 2.2 μL of 250 mM piperidine and 250 mM imidazole in 80:20 acetonitrile/water.[32,33] Calibration of the analyzer, in negative-ion mode, used a synthetic DNA oligomer (5′-AGTTTGGTTAAGTTAGAA-3′, MW 5,592.7). The ESI source was modified by removing the standard pepper pot used for megaflow on-line liquid chromatography MS analysis.[13] The nebulizer was operated at a flow rate of 4 μL/min, with sample introduction using a 25-μL Hamilton syringe. A capillary voltage of ∼2.55 kV was applied to produce stable ionization. A minimum of 100 scans were collected at 29 scans/min. The cone voltage was set at 40 V.

Analysis was also performed using a Finnigan LCQ quadrupole ion trap (Finnigan, San Jose, California). Acetonitrile and piperidine/imidazole were added to each sample—50% and 25 mM final concentration, respectively. A hand-pulled fused silica glass capillary (180-μm o.d. and 50-μm i.d.) was used as the ESI tip by threading through the metal capillary of the LCQ source block and a 3.5 kV potential applied at the syringe needle. A SF_6 sheath gas flow was used to reduce corona discharge. The LCQ has an external unheated nebulizer, and a short heated inlet capillary carries charged species into the interface. A heater was attached to the syringe of a Hamilton syringe pump, used for sample transfer to the nebulizer, to dissociate the ds PCR product. The sample was kept at an elevated temp within the syringe using a 1" long, 1/8" diameter cartridge heater from Omega (Stamford CT). The heater was driven at 120 V and manually controlled to maintain a solution temp just below boiling. The LCQ was operated using a heated capillary temperature of 180 °C and 7% relative source collision energy. For each scan, three microscans were performed at 200 milliseconds each. A minimum of 100 scans were collected at 30 scans/min for each product ion spectrum. Parent ions were isolated with a 7 m/z window, and dissociations were performed with the relative collision energy at 20−25%. He pressure in the trap was maintained at $(1-2) \times 10^{-5}$ Torr.

For quadrupole analysis, each sample contained a total of 25 pmol of DNA. For the quadrupole ion trap, 2 pmol was analyzed. The difference in amount of DNA analyzed relates primarily to the flow rate for analysis (300 nL/min for the LCQ and 4 μL/min for the Quattro). Acquisition time was approximately 3 min total for each.

Fragment peaks were identified using the program DFCalc (University of Washington, Seattle, Washington). DFCalc was written to generate a list of ions that could be expected to form from the fragmentation of known DNA sequences. The user interface allows the entry of the molecular sequence. Additionally, the ion types of interest, the mass and charge-state ranges of those ions, and the inclusion of double fragmentation events can all be selected. The ion lists are directed to a text file that includes DFcalc settings and a table with the m/z, mass, charge, type, and sequence of each ion. The freeware can be obtained at the following URL: http://fields.scripps.edu/DFcalc.

RESULTS AND DISCUSSION

It is presented here that MS analysis of two PCR products allows the discrimination of SNPs by the determination of mass differences. More complex studies, in which two SNPs occur in a single stretch of DNA, can be more readily distinguished using MS/MS analysis. Differentiation of two isomeric PCR products having the same mass, but different sequences is used as an example of such an analysis.

Figure 1 compares ESI quadrupole MS spectra of ds 69 bp PCR products, containing a C at position 38 (+ strand) and G at position 32 (− strand) (Figure 1A) or a G at position 38 and a C

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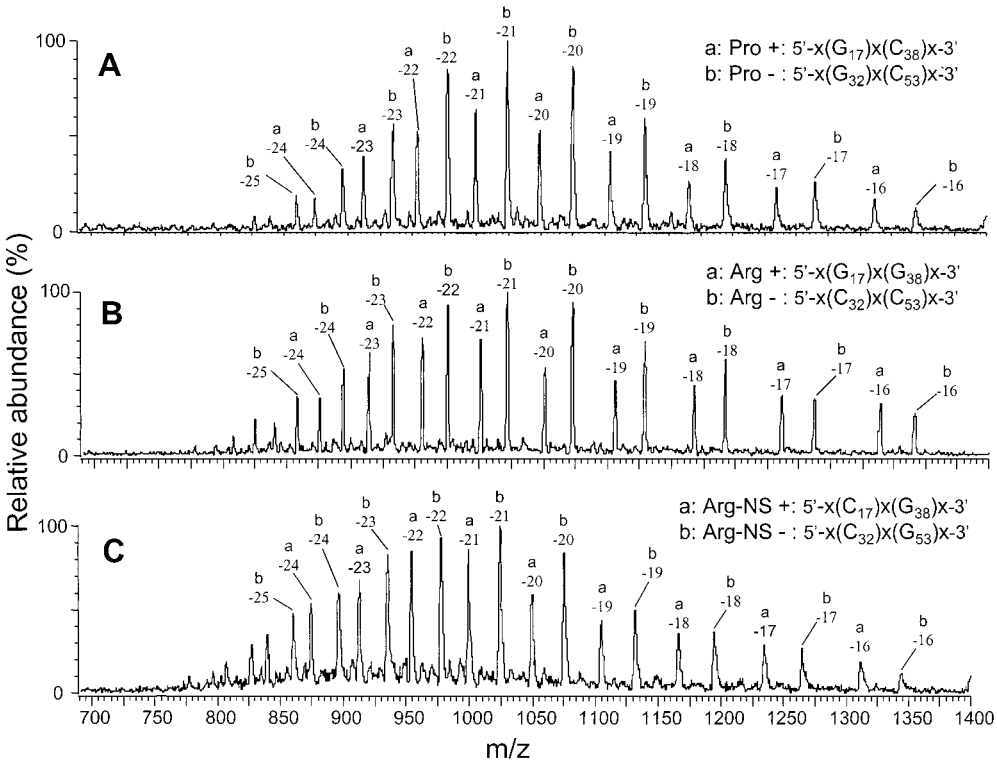

Figure 1. ESI quadrupole mass spectra of 69 bp ds PCR products of the p53 gene. (A) pro, (B) arg, and (C) arg-NS variants.

at position 32 (Figure 1B). All nucleotide positions are numbered from the 5′-3′ direction. The C or G polymorphism in the + strand would result in an increase of 40.0 Da, with the corresponding change within the − strand of (−)40.0 Da. The measured MWs for all 4 strands were within 4.4 Da of the predicted, with a measured difference of (+)39.4 Da for the + strands (pro [C] versus arg [G]) and (−)40.0 Da for the − strands (pro [G] versus arg [C]). The third PCR product, arg-NS (Figure 1C), contains an additional G→C switch which negates the polymorphism, resulting in an identical MW as the pro variant of p53, but with a different sequence. The + strand of the PCR product has a G at the polymorphic site (nucleotide position 38), like the arg variant, but a C at nucleotide 17 [5′− $x(C_{17})x(G_{38})x$ − 3′]. Figure 1C (arg-NS) cannot be distinguished from Figure 1A (pro). The differences in measured MWs between the pro and arg-NS PCR products were within 5.1 Da for both strands.

To discriminate SNPs or other changes in sequence by MS it is essential that PCR products be denatured and the mass of the single strands be determined. Otherwise, mass changes in one strand (e.g., G→C) are neutralized by mass changes on the complementary strand (e.g., C→G).[11,34] It was noted with the ESI quadrupole source (Micromass Quattro 1), ds PCR products were inherently denatured into the two complementary strands. A metal nebulizer probe inserted into a heated source (approximately 100 °C) readily transfers heat from the source to the probe. To consistently obtain ss PCR products on the ESI quadrupole ion trap (Finnigan LCQ), on-line heating with a modified source, as described in the materials and methods, was required. Figure 2 clearly shows that, without heating, the pro ds PCR product is observed as a mixture of dissociated single-strand and intact DNA duplexes. However, on heating, only single strands are observed. The calculated MW of the ionized ds PCR product was 42 585 ± 1.8 Da, 62.6 Da more than predicted. The insets of Figure 2 show that nonadducted (m/z 1131.5) and monoadducted species (m/z 1132.7) are observed in both spectra, whereas the disodium adducted species (m/z 1134.2) does not appear in the heated sample (Figure 2B). Therefore, the ESI quadrupole ion trap mass spectrometer required both on-line heating and microdialysis for optimal analyses. ESI quadrupole ion trap MS also readily distinguished the C→G polymorphism. The measured MW for all four strands was within 3.8 Da of that predicted, with a measured difference of (+)38.9 Da for the + strands and (−)41.0 Da for the minus strands. The pro and arg-NS PCR products were indistinguishable as expected.

The ESI quadrupole ion trap in MS/MS mode was successfully used to discriminate all 3 human p53 69 bp PCR variants (see Figure 3 and Table 1). The nomenclature of McLuckey is used.[35,36] Ions a−d are numbered from the 5′ end while the corresponding ions w−z are numbered from the 3′ end; in all cases, breakage occurs at a phosphodiester bridge. Secondary ions can be generated by an additional break in the primary fragment, also at a phosphodiester bridge, creating an internal fragment. Five ions identified from the product ion spectra of the minus strand (−21 charge state) were derived from the sequence containing the NS (nucleotide position 53). These consist of three w ions (w20, w22, and w27) and two possible internal fragments ([a63-Cyt]→z26 and [b62-Thy]→y30), all with charge states of −7 except for w27 which

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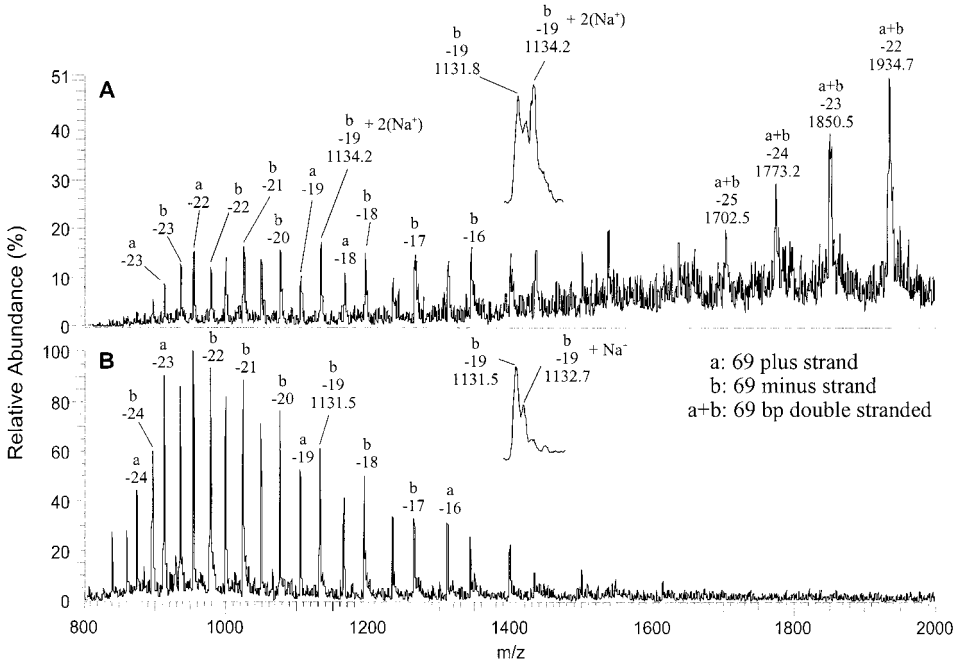

Figure 2. ESI quadrupole ion trap mass spectra of the 69 bp ds PCR products of p53 (pro variant) (A) unheated and (B) heated.

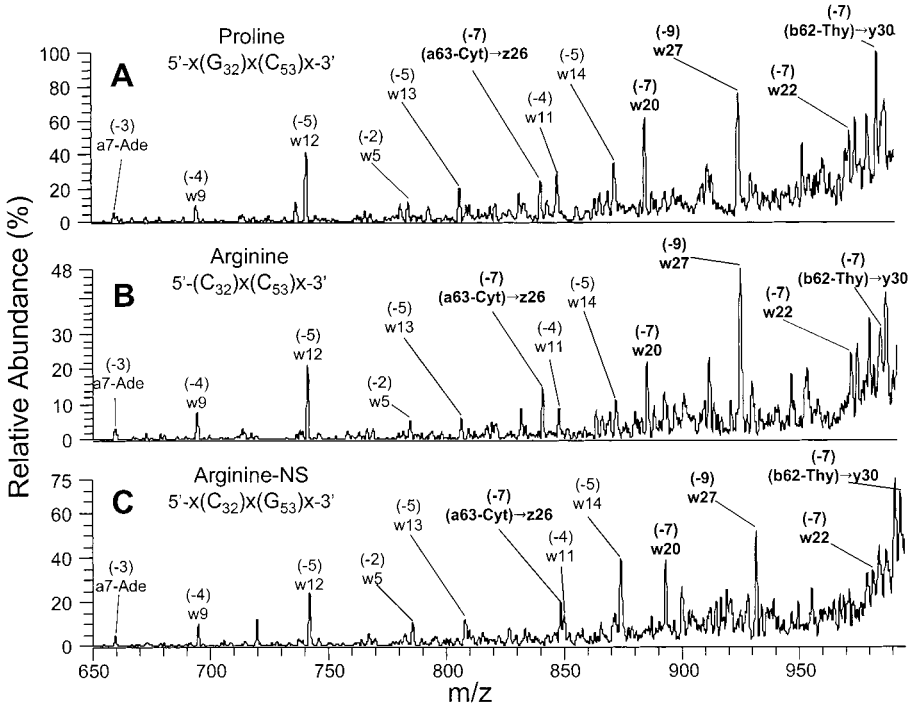

Figure 3. ESI quadrupole ion trap product ion mass spectra of the minus strand (−21 charge state) of three 69 bp PCR products. (A) pro, (B) arg, and (C) arg-NS variants of p53.

was −9. The measured difference of these fragments between the pro and the arg-NS PCR products ranged from (+)38.5 to (+)39.9. Unfortunately, no ions were found which contained the polymorphism (nucleotide position 32). This might be explained by the polymorphism being at position 32 (central), whereas the negating switch is at position 53 (17 bases from the terminus).

To obtain product ion spectra that would contain both the polymorphism and the negating switch, the 69 bp PCR products were digested with Hae III. Using ESI quadrupole MS analysis, two products predominated, 26 bp and a 43 bp, with the 43 bp fragment containing both switches. The terminal phosphates were present at the 5′ end of each strand at the cut site. The measured MW of all strands was within 4.2 Da of the predicted.

Several fragment ions of the + strand (−13 charge state) incorporated sequence differences between pro and arg variants as seen in the product ion spectra (see Figure 4 and Table 2). Seven identified ions contained the polymorphism, position 38 (C or G), and two ions contained the NS, position 17 (G or C). Additionally, four ions contained both the polymorphism and the NS, and three ions contained neither, all of which were indistin-

Table 1. Predicted (Pred) and Measured (Meas) m/z and MW of Fragment Ions from Product Ion Spectra (ESI Quadrupole Ion Trap)[a]

	pro		arg		arg-NS			measured	measured	predicted	predicted
	5′−x(G$_{32}$)x(**C$_{53}$**)x-3′		5′−x(C$_{32}$)x(C$_{53}$)x-3′		5′−x(C$_{32}$)x(**G$_{53}$**)x-3′			shift	shift	switch	shift
product ion	pred *m*/*z*	meas *m*/*z*	pred *m*/*z*	meas *m*/*z*	pred *m*/*z*	meas *m*/*z*	charge state	pro→arg-NS *m*/*z*	pro→arg-NS (Da)	pro→arg-NS (Da)	pro→arg-NS (Da)
a7-Ade[b]	660.1	660.0	660.1	660.0	660.0	659.9	(−3)	(−)0.1	(−)0.3	none	0.0
w9	695.2	695.2	695.2	694.9	695.2	695.1	(−4)	(−)0.1	(−)0.4	none	0.0
w12	742.3	742.1	742.3	742.1	742.3	742.2	(−5)	(+)0.1	(+)0.5	none	0.0
w5	786.0	785.9	786.0	786.1	786.0	785.9	(−2)	0.0	0.0	none	0.0
w13	808.1	808.0	808.1	807.7	808.1	808.0	(−5)	0.0	0.0	none	0.0
(a63-Cyt[c])→z26[d]	**842.8**	**842.7**	**842.8**	**842.5**	**848.6**	**848.3**	**(−7)**	**(+)5.6**	**(+)39.2**	**C→G**	**(+)40.0**
w11	849.8	849.7	849.8	849.7	849.8	849.8	(−4)	(+)0.1	(+)0.4	none	0.0
w14	874.0	874.0	874.0	873.9	874.0	873.8	(−5)	(−)0.2	(−)1.0	none	0.0
w20	**887.4**	**887.3**	**887.4**	**887.3**	**893.1**	**892.9**	**(−7)**	**(+)5.6**	**(+)39.2**	**C→G**	**(+)40.0**
w27	**927.2**	**927.2**	**927.2**	**927.3**	**931.6**	**931.5**	**(−9)**	**(+)4.3**	**(+)38.7**	**C→G**	**(+)40.0**
w22	**975.8**	**975.4**	**975.8**	**975.0**	**981.5**	**981.1**	**(−7)**	**(+)5.7**	**(+)39.9**	**C→G**	**(+)40.0**
(b62-Thy[e])→y30[d]	**987.5**	**987.5**	**987.5**	**987.6**	**993.2**	**993.0**	**(−7)**	**(+)5.5**	**(+)38.5**	**C→G**	**(+)40.0**

[a] The minus strands (−21 charge state) of 69 bp PCR products were analyzed. [b] Adenine. [c] Cytosine. [d] Possible internal fragment. [e] Thymidine.

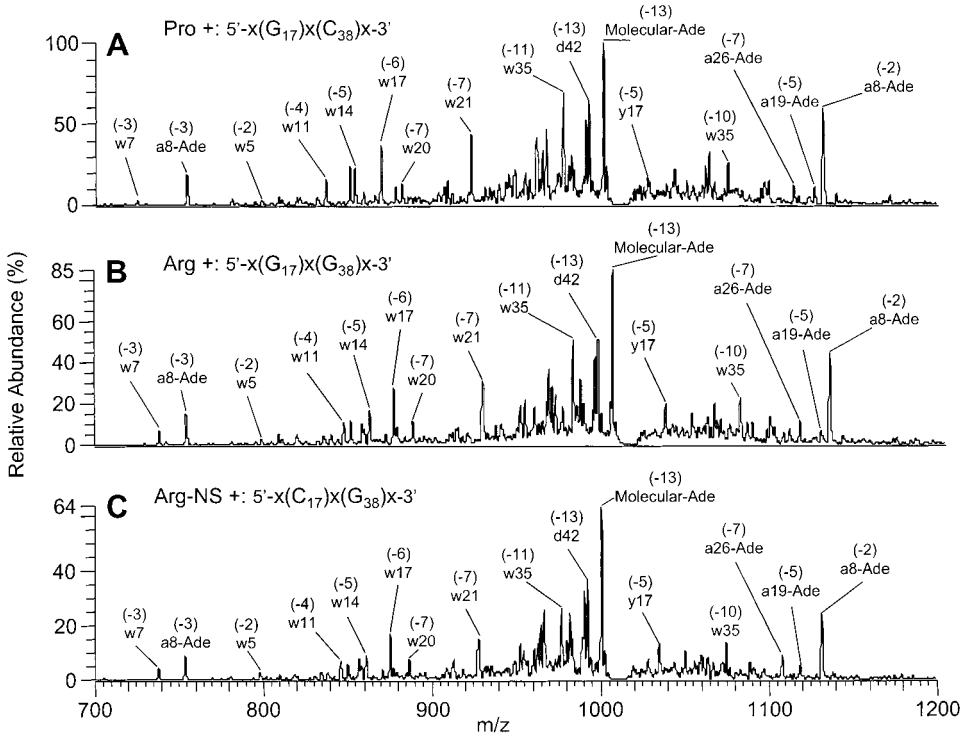

Figure 4. ESI quadrupole ion trap product ion spectra of plus strand (−13 charge state) 43 bp component of restriction-digested p53 PCR products. (A) pro, (B) arg, and (C) arg-NS variants.

guishable across the three spectra. All the measured *m*/*z* ratios were within 0.5 *m*/*z* of the predicted. The charge states of each of the identifiable fragments ranged from −13 to −2 (Table 2). Thus, MS/MS fragmentation of 43 bp restriction fragments using the ESI quadrupole ion trap allowed unequivocal differentiation of PCR products with the same nucleotide composition but different sequences. As predicted, product ion spectra of the 26 bp restriction fragment minus strands (−7 charge state) using the ESI quadrupole ion trap were virtually identical.

CONCLUSIONS

A model system was employed using plasmids encoded with the arg and pro variants of p53. Additionally PCR directed mutagenesis was used to generate a PCR product exhibiting a more complex genetic change (NS). As demonstrated here, both quadrupole and quadrupole ion trap MS analysis allow discrimination of PCR products differing by an SNP. However, regions of human DNA from different individuals can contain more complex differences (including multiple SNPs, rearrangements, and insertions/deletions). Discrimination of two isomeric PCR products (same mass and nucleotide composition but different sequences) was also demonstrated here using quadrupole ion trap MS/MS.

At the current time, ESI triple quadrupole MS/MS is limited to relatively small oligonucleotides.[25] Nozzle-skimmer fragmentation has proved necessary to produce dinucleotide primary ions. MS/MS analysis of secondary ions (mononucleotides) then

Table 2. Predicted (Pred) and Measured (Meas) *m*/*z* and MW Identified from Product Ion Spectra (ESI Quadrupole Ion Trap)[a]

	variants										
	pro		arg		arg-NS						
	5′-*x*(G_{17})-*x*(C_{38})*x*-3′		5′-*x*(G_{17})-*x*(G_{38})*x*-3′		5′-*x*(C_{17})-*x*(G_{38})*x*-3′			measured shift	measured shift	predicted switch	predicted difference
product ion	pred *m*/*z*	meas *m*/*z*	pred *m*/*z*	meas *m*/*z*	pred *m*/*z*	meas *m*/*z*	charge state	pro→arg-NS *m*/*z*	pro→arg-NS (Da)	pro→arg-NS	pro→arg-NS (Da)
w7	724.8	724.8	738.1	738.1	738.1	738.1	(−3)	(+)13.3	(+)39.9	C→G	(+)40.0
a8-Ade[b]	754.2	754.2	754.2	754.1	754.2	754.1	(−3)	(−)0.1	(−)0.3	none	0.0
w5	798.5	798.4	798.5	798.5	798.5	798.4	(−2)	0.0	0.0	none	0.0
w11	836.3	836.3	846.3	846.3	846.3	846.1	(−4)	(+)9.8	(+)39.2	C→G	(+)40.0
w14	853.3	853.3	861.3	861.5	861.3	861.3	(−5)	(+)8.0	(+)40.0	C→G	(+)40.0
w17	868.9	868.9	875.6	875.7	875.6	875.5	(−6)	(+)6.6	(+)39.6	C→G	(+)40.0
w20	881.1	881.1	886.9	886.8	886.9	886.9	(−7)	(+)5.8	(+)40.6	C→G	(+)40.0
w21	922.4	922.4	928.2	927.7	928.2	927.9	(−7)	(+)5.5	(+)38.5	C→G	(+)40.0
w35	977.1	977.1	980.7	980.7	977.1	977.0	(−11)	(−)0.1	(−)1.1	G→C/C→G	0.0
d42	992.1	992.2	995.2	995.3	992.1	992.2	(−13)	0.0	0.0	G→C/C→G	0.0
molecular-Ade	1000.9	1000.8	1004.0	1003.9	1000.9	1000.8	(−13)	0.0	0.0	G→C/C→G	0.0
y17	1026.9	1026.8	1034.9	1034.9	1034.9	1034.8	(−5)	(+)8.0	(+)40.0	C→G	(+)40.0
w35	1074.9	1074.9	1078.9	1078.7	1074.9	1074.7	(−10)	(−)0.2	(−)2.0	G→C/C→G	0.0
a26-Ade	1114.0	1113.9	1114.0	1113.8	1108.3	1108.1	(−7)	(−)5.8	(−)40.6	G→C	(−)40.0
a19-Ade	1126.5	1126.3	1126.5	1126.3	1118.5	1118.5	(−5)	(−)7.8	(−)39.0	G→C	(−)40.0
a8-Ade	1131.7	1131.3	1131.7	1131.4	1131.7	1131.4	(−2)	(+)0.1	(+)0.2	none	0.0

[a] The restriction fragments of the + strand of a 43 bp were analyzed. [b] Adenine.

provided structural information.[25] As presented here, ESI quadrupole ion trap MS/MS analysis of higher mass PCR products and their restriction fragments is feasible. This might be anticipated on the basis of theoretical considerations of instrument design ("tandem in time" versus "tandem in space").[37] It has been noted that ion trap detectors, in general, offer higher sensitivities in product ion mode, but when analyzing "real samples" the value of the spectral information might be limited by interference of matrix ions. However, the greatly increased sensitivity of the ion trap for generating product ion spectra has also been experimentally demonstrated for trace detection in a variety of complex clinical and environmental matrixes.[38]

McCloskey and co-workers first demonstrated that nucleotide composition could be determined by MW analysis using quadrupole MS analysis.[39] However, the number of possibilities rapidly increases as mass increases. More accurate molecular weight assignment, using FTICR MS analysis, restricts the possible nucleotide composition as chain length increases.[40,41] Using stable-isotope-labeled dNTP precursors, the nucleotide composition of restriction digests of PCR products can also be determined on MALDI-TOF MS.[42] However, two PCR products with the same nucleotide composition, but different sequences (e.g., negating switch) will, of course, have the same mass on MS analysis and cannot be discriminated regardless of the configuration of the MS instrument employed. As demonstrated here, MS/MS is a powerful approach for discrimination of two PCR products of the same nucleotide composition (and mass) but different sequence.

It is not suggested that the methodology described here could be used for population studies of genetic diversity. For example, high-throughput population studies will certainly involve the analysis of genomic DNA, not plasmids. However it might be useful to consider how a mature mass spectrometry technology might be used. In studying a group of unknowns, it would be required that the sequence of the region of interest first be known for one "standard human genomic DNA". Primers would then be designed against this known sequence. The masses of each PCR product (which would not have been previously sequenced) would be determined using MS. If the masses fall into two clear groups, a polymorphism would have been identified. The use of MS/MS would then be used as a complementary technique, to determine the presence of genetic differences other than an SNP. Some of these changes might result in regions with the same nucleotide composition and mass but different sequence. Such changes would be missed on MS analysis. Most product ion spectra would fall into two patterns representing the SNP. The purpose would be to identify aberrant PCR products that did not fall into these two fragmentation patterns. In most instances, these PCR products would represent only a small portion of samples. Only PCR products with aberrant MS/MS fingerprints would then be subject to conventional Sanger sequencing. Prior MS and MS/MS analysis, used as a screening technique, could drastically reduce the amount of sequencing in genomic population studies. In the current report, our assignment of peaks in product ion spectra was merely to demonstrate that the fragmentation patterns are interpretable on the basis of known sequence. However, in a

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population study, it might not be necessary to identify all, or indeed any, peaks in product ion spectra.

In summary, MS and MS/MS are complementary techniques. MS analysis (using quadrupole or ion trap instruments) allows discrimination of single nucleotide polymorphisms. Quadrupole ion trap MS/MS allows differentiation of PCR products of different sequence, even if they have the same nucleotide composition. The combination of MS and MS/MS has great potential for use in the study of diversity of the human genome.

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