BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 173–177 (1997)
ARTICLE NO. RC976925

Qualitative and Quantitative Catalog of Tyrosinase Alternative Transcripts in Normal Murine Skin Melanocytes as a Basis for Detecting Melanoma-Specific Changes

Stephen R. Kelsall, Nathalie Le Fur, and Beatrice Mintz[1]
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received June 2, 1997

The decline in cell differentiation commonly associated with malignant progression may be due in part to an increase in alternative splicing of the pre-mRNAs of tissue-specific genes. As a necessary basis for investigating this possibility in a murine model of cutaneous melanoma, the complete qualitative and quantitative inventory of alternative transcripts was sought for the tyrosinase gene in normal mouse skin melanocytes, as this gene plays a key role in melanization. Of 111 alternative mRNAs predicted from known splice sites in the gene, 19 isoforms were detected, and their abundances determined, through a systematized protocol involving splice junction-specific probes, exon-specific restriction enzymes, and quantitative RT-PCR with an RNA internal standard. No unpredicted tyrosinase transcripts were discovered. Two of the transcripts, each involving an intra-exonic deletion and present at relatively low abundance in normal skin, were subsequently found to be consistently upregulated in melanomas.

The gradual decline in cell differentiation which is a common feature of malignant tumors (1) implies that the expression of tissue-specific genes is changing. The change may be due in part to a selective increase in alternative splicing of some pre-mRNAs of these genes, as the proteins generated from splice variants would often lack the function of proteins produced from the constitutively spliced mRNAs. Some of the novel proteins might nevertheless play important biological roles in tumors, particularly if any of the peptides resulting from intracellular proteolysis have immunostimulatory properties (2).

Melanization is the tissue-specific phenotype of normal melanocytes. Melanomas tend to become hypomelanotic or amelanotic, hence less differentiated, as they progress in malignancy (3). Of the many genes known to contribute to pigment formation (4), tyrosinase encodes the rate-limiting enzyme catalyzing the initial steps in melanin biosynthesis (5). The present investigation of tyrosinase alternative splice variants in normal pigment cells of C57BL/6 mouse skin was undertaken as a basis for detecting any significant splicing changes in malignant cutaneous melanomas from transgenic mice of the same inbred strain (6, 7). The tyrosinase gene has been isolated and some of its pre-mRNA splicing possibilities described (8-10).

A systematic scheme was needed to ensure that all tyrosinase transcripts would be recognized and their levels correctly assessed, especially as any specific mRNA increases in melanomas might pertain to isoforms with only minor representation in normal skin. We describe here the protocol designed for this purpose, its application to murine tyrosinase to obtain the complete qualitative and quantitative transcript profile in skin melanocytes, and the relevance of the results for melanomas.

MATERIALS AND METHODS

Cellular and internal standard RNAs. Approximately 100 mg of dorsal body skin from 8-day-old C57BL/6 mice (Icr subline) was subjected to the guanidinium thiocyanate-phenol-chloroform extraction procedure of Chomczynski and Sacchi (11). The extracted RNA was dissolved in RNAse-free water at a concentration of 1 μg/μl.

An RNA internal standard was constructed by making a 98-bp deletion (nt 292-391) in the tyrosinase cDNA (9) in pBluescript (Stratagene), and then synthesizing the RNA *in vitro* from the T7 promoter after cleaving with *Hpa*I (nt 2032) to prevent run-on of the polymerase.

Reverse transcription-polymerase chain reaction (RT-PCR). Sample RNA (2.5 μg), with or without the internal standard RNA, was

[1] To whom correspondence should be addressed: Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. Fax: (215) 728-3574.

Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction.

0006-291X/97 $25.00

reverse-transcribed with SuperScript II according to instructions of the manufacturer (GIBCO BRL), with the use of a tyrosinase-specific primer (nt 1721-1701). The reaction volume was 40 μl and the reaction temperature was 45°C.

Primers for PCR were complementary to nt 56-81 and nt 1674-1650, which respectively overlap the tyrosinase start and stop codons. Reaction mixtures (75 μl) contained 200 ng of reverse-transcribed RNA and PCRs were initiated by a wax-mediated hot-start procedure (12). Final buffer conditions followed instructions of the polymerase manufacturer (Ampli*Taq*, Perkin-Elmer), with the $MgCl_2$ at 1.5 mM concentration. All PCRs were carried out for 25 cycles, thereby maintaining the reaction within the exponential phase (as described below). The first two rounds of PCR were at 95°C for 3 min, 62°C for 1 min, and 72°C for 90 s; ensuing cycles were at 95°C for 1 min as the first step. Following the final cycle, tubes were held at 72°C for 7 min.

Oligonucleotide probes. The following splice junction-specific probes were synthesized; the relevant junction is indicated in parentheses, with the nucleotide sequence numbering according to Ref. 9: GTGGATGACCGCACCTATGGC (291/522), GTGGATGACCATCATTTGTAG (291/881), CTGCGGAAACTCATCCTTCTT (369/858), CTGCGGAAACTATCATTTGTA (369/881), CTGCGGAAACTGCACCTATGG (369/522), GTGGATGACCCATCCTTCTTC (291/858), GTGGATGACCGATTTGCCAGT (291/1098), GTGGATGACCTATTTTTGAAC (291/1246), GGTGGATGACCATCCAGGCTT (291/1428), CTGCGGAAACTGATTTGCCAG (369/1098), CTGCGGAAACTTATTTTTGAA (369/1246), CTGCGGAAACTATCCAGGCTT (369/1428). Probes were also synthesized that were specific for continuous stretches of sequence, as follows: prΔ2-4, nt 495-515; prEx3, nt 1129-1110; prTyr, nt 1665-1644; and prA1, AGACTGCATGGTGCGAAAGA (representing an unpublished sequence in the A1 insert).

Determination of exponential phase of the PCR and quantitation of transcripts. To locate the exponential phase, the reaction products from successive PCR cycles were analyzed by Southern hybridization (13) with the prTyr probe, which was designed to recognize all transcripts (including the internal standard). The intensities of the bands corresponding to the full-length and the alternative transcripts were measured with a BAS1000 phosphorimager (Fuji). A log-linear plot showed the same exponential rate of increase (~1.8) for all intensities until cycle 26.

Most amplified transcripts migrated through agarose so that each yielded a single homogeneous band, e.g., Δ1a, Δ1b, Δ1d, Δ1e,3, and Δ3,4. To quantitate each of these, the blots were probed with prTyr and the intensity of the relevant band was directly compared with that of the internal standard band. However, the Δ1e, Δ3, and Δ4 transcripts formed a triplet of comigrating components requiring individual identification. To measure the Δ1e transcript, the prEx3 probe was used, as this did not hybridize with the Δ3 isoform but still recognized the internal standard; *Apa*I digestion (cutting at nt 386) removed the Δ4 transcript. The Δ3 and Δ4 transcripts were measured together as a single band in isolation from Δ1e through the use of probe prΔ2-4. The value obtained was apportioned to the Δ3 and Δ4 components by application of the ratio 95.5/4.5 ± 0.8 (n=3), which was first determined by measuring the Δ3 and Δ4 bands after their separation by *Bst*XI digestion (at nt 1387). The comigrating full-length and A1 transcripts were measured after separation by *Sca*I digestion (at nt 811). Internal standard was generally used at 100 fg (8.8×10^4 molecules) per μg cellular RNA but was at 10 fg per μg for the Δ1e measurements.

RESULTS AND DISCUSSION

Detection of alternative transcripts. As a starting point, all possible alternative mRNAs of tyrosinase were predicted from the splice sites utilized to generate the twelve known splice variants (8-10). When all combinations of splice sites were considered, 111 alternative transcripts were possible. A strategy was then devised that would unequivocally identify each of these transcripts, if present. This was based on analysis of RT-PCR products with a combination of specific oligonucleotide probes and restriction enzymes. Twelve splice junction-specific probes and two probes specific for continuous sequence (prΔ2-4 and prA1) were used on Southern blots, with most probes recognizing between 1 and 16 potential transcripts. Three restriction enzymes were employed, singly or in combination; each was specific for one of the three internal exons and served to test for the absence of that exon.

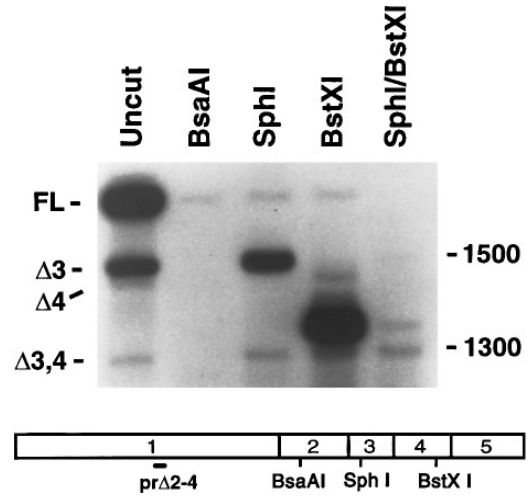

FIG. 1. An example of the combined use of a specific probe and of exon-specific enzymes to identify alternative mRNA isoforms of tyrosinase. In the map are indicated the site of hybridization of the probe (prΔ2–4) and the locations of selected restriction sites in exons 2, 3, and 4. The Southern blot shows hybridized DNA in the uncut form, after single digestion with the designated three enzymes, and after double digestion with two of them. Bands are identified at the left. Positions of size markers (in base pairs) are at the right.

The example in Fig. 1 documents the use of probe prΔ2-4 with the exon-specific enzymes. This probe was designed to detect the 16 transcripts (including the full-length one) that would have no deletion in exon 1. Hybridization of RT-PCR products revealed the full-length transcript and three additional bands which apparently represented splice variants (Fig. 1, "uncut" lane). The sizes of the cDNAs indicated their identities as Δ3, Δ4, and Δ3,4 transcripts, which would respectively lack exon 3, exon 4, and both exons 3 and 4 (with the transcript nomenclature according to Ref. 10). Restriction digestion confirmed the initial identification: all bands were absent from the *Bsa*AI lane, since this enzyme cuts in exon 2; the Δ3 band was present in the *Sph*I lane, since this enzyme cuts in exon 3; the Δ4 band was present in the *Bst*XI lane, since this enzyme cuts in exon 4; and the Δ3,4 band was present in all lanes except that of *Bsa*AI.

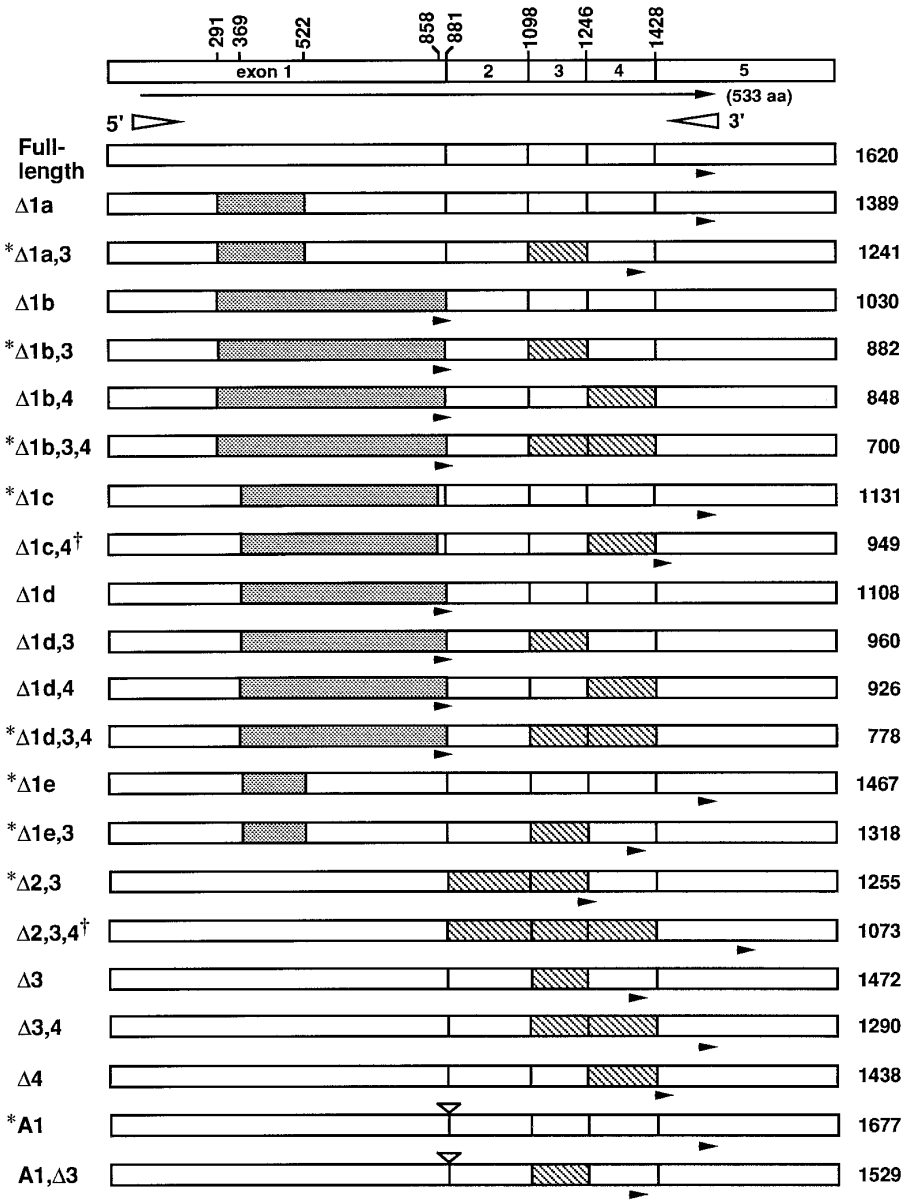

FIG. 2. Tyrosinase alternative transcripts. The map at the top represents the exon structure of the full-length mRNA, the positions of the alternative splice sites in exon 1, and the location of the tyrosinase coding sequence (arrow). The two open arrowheads indicate the positions of the PCR primers. Underneath are shown the full-length (constitutive) transcript and the 19 alternative transcripts that were identified. Splice variants not previously reported are preceded by an asterisk. Two transcripts found in earlier work but not in this study are marked by daggers. Deletions in exon 1 are indicated by shading, skipped exons by cross-hatching; a triangle denotes the A1 insert (57 nt). The size (in base pairs) of the RT-PCR product of each transcript is at the right. The solid arrowhead beneath each transcript marks the stop codon of the reading frame. All reading frames were assumed to have the same initiation codon as that of the full-length transcript.

Specific alternative transcripts of tyrosinase in normal mouse skin melanocytes. When the analysis was complete, 19 alternative isoforms had been found (Fig. 2), of which nine (marked with asterisks) had not previously been detected. Two transcripts (marked with daggers), reported earlier (10), were not seen in the present study but have been included for completeness; they may have gone undetected due to the markedly lower number of PCR cycles used here than in the earlier study (25 *versus* 50 cycles). All the recognized alternative transcripts were products of skipping of one or more internal exons, deletion within exon 1, and/or inclusion of a short, presumably intronic, sequence (A1) between exons 1 and 2. Of the six potential deletions in exon 1, only one, tentatively named Δ1f (splice donor at nt 291, splice acceptor at nt 858), was not detected. Even with the prTyr probe, designed to recognize all amplified transcripts, no evidence was found for pres-

TABLE 1

Levels of Tyrosinase Alternative Transcripts in Skin Melanocytes

Transcript(s)	Molecules/ng RNA[a]	Full-length (FL) transcript (%)
FL	326 ± 26	100
Δ3	40.7 ± 4	12.5
Δ1e	10.8 ± 1.5	3.3
Δ1a, Δ1d, Δ4, Δ1b, Δ3,4[b]	2.6 ± 0.3–1.5 ± 0.2	0.8–0.5
A1	1.2 ± 0.2	0.3
Δ1e,3	0.02 ± 0.02	0.07
Others[c]	<0.2 ± 0.02	<0.07

[a] Intensities of transcript bands on Southern blots were compared with the intensity of the RNA internal standard band, present at 88 or 8.8 molecules/ng cellular RNA. Comigrating isoforms were separated by restriction digestion and hybridization with specific probes (see Materials and Methods). Data are means ± S.D. ($n = 3$).

[b] Order of listing is from highest to lowest abundance.

[c] Δ1a,3, Δ1b,3, Δ1b,4, Δ1b,3,4, Δ1c, Δ1d,3, Δ1d,4, Δ1d,3,4, Δ2,3, A1, Δ3.

ence of alternative transcripts other than those included among the 111 predicted ones. Thus, the catalog of transcripts (Fig. 2) represents the complete array of alternative isoforms for the normal tyrosinase coding sequence, with the possible exception of very rare transcripts detectable only with high PCR cycle numbers.

The levels of the most abundant mRNA isoforms are listed in Table 1. Lower-abundance alternative species are grouped. Δ3 was the most highly represented splice variant, followed by Δ1e.

Polypeptide products of tyrosinase splice variants in normal mouse skin melanocytes. The deduced proteins encoded by the tyrosinase alternative transcripts extend from the tyrosinase start codon to the filled arrowheads in Fig. 2. These proteins, which would generally be expected to lack the normal function of tyrosinase, would have varying lengths of sequence that is absent from the protein encoded by the constitutive isoform. Redundancy occurs in a number of the "alternative" protein sequences. For example, when exon 3 is skipped, the 45-amino-acid alternative sequence encoded by the downstream RNA would be present in the proteins encoded by the Δ3, Δ1a,3, Δ1e,3, and A1,Δ3 transcripts. Due to redundancy, all of the "alternative" protein sequences encoded by the 19 alternative transcripts are represented in just 10 transcripts: Δ1a, Δ1b, Δ1c, Δ1d, Δ1e, Δ3, Δ4, Δ2,3, Δ3,4, and A1.

Comparisons of tyrosinase alternative transcripts in normal mouse skin melanocytes and melanomas. Knowledge of the tyrosinase transcript inventory in normal melanocytes now enables changes to be detected in various melanocytic diseases. Subsequent to the present study, the data described here were utilized in the analysis of a large sample of malignant mouse melanomas experimentally produced in transgenic mice (7). Comparison with normal skin melanocytes of the same inbred strain revealed the same tyrosinase transcripts as in skin. However, an overall increase in alternative splicing of the tyrosinase pre-mRNA had occurred in the tumors and the Δ1b and Δ1d splice variants were specifically elevated over their very low levels in skin (14). The increases were especially marked in the early (melanotic) stage of the primary tumors. As malignancy progressed and the tumors became amelanotic, overexpression of these specific mRNAs was less pronounced; in a few cases, tyrosinase transcription ceased. The selective increases in Δ1b and Δ1d mRNAs represent increased usage of two internal splice donor sites in exon 1, at nt 291 and nt 369, in conjunction with shared usage of the constitutive splice acceptor site at the 5′ end of exon 2 (Fig. 2). Levels of the other splice variants within that exon (Δ1a, Δ1c, and Δ1e) did not appreciably change. The results provide a basis for predicting novel peptides of possible immunotherapeutic value from overexpressed splice variants in the tumors. After translation of these mRNAs and proteolytic processing of the proteins, some of the candidate peptides may form complexes with major histocompatibility proteins that are capable of eliciting a cytotoxic T-lymphocyte response against the tumor cells (15, 16). An antigenic peptide of a melanocytic gene that was recently found in human melanoma (2) may in fact have arisen via translation of an alternatively spliced mRNA.

The preferred alternative splicing choices of tyrosinase in normal *versus* malignant melanocytes may help to clarify the mechanisms of splicing control (17-19) in the normal cells as well as their changes in malignancy. It will be of interest to obtain comparative mRNA inventories of other melanocytic genes in normal and malignant pigment cells. A broader study of relevant tissue-specific normal genes in other kinds of malignancies may reveal whether consistent splicing modifications, without mutation in the genes of interest, are a general attribute of malignant conversion.

ACKNOWLEDGMENTS

This work was supported by an American Cancer Society Special Research Grant for Metastatic Melanoma (NP-917) and a Johnson & Johnson Focused Giving Grant to B.M., and by National Institutes of Health Grants CA-06927 and RR-05539 to the Fox Chase Cancer Center.

REFERENCES

1. Mintz, B. (1978) *Harvey Lect.* **71,** 193–246.
2. Wang, R.-F., Parkhurst, M. R., Kawakami, Y., Robbins, P. F., and Rosenberg, S. A. (1996) *J. Exp. Med.* **183,** 1131–1140.
3. McGovern, V. J. (1983) Melanoma: Histological Diagnosis and Prognosis, Raven, New York.

4. Silvers, W. K. (1979) The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction, Springer, New York.
5. Hearing, V. J., and Jimenez, M. (1987) *Int. J. Biochem.* **19,** 1141–1147.
6. Bradl, M., Klein-Szanto, A., Porter, S., and Mintz, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 164–168.
7. Mintz, B., and Silvers, W. K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8817–8821.
8. Ruppert, S., Müller, G., Kwon, B., and Schütz, G. (1988) *EMBO J.* **7,** 2715–2722.
9. Terao, M., Tabe, L., Garattini, E., Sartori, D., Studer, M., and Mintz, B. (1989) *Biochem. Biophys. Res. Commun.* **159,** 848–853.
10. Porter, S., and Mintz, B. (1991) *Gene* **97,** 277–282.
11. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
12. Chou, Q., Russell, M., Birch, D. E., Raymond, J., and Bloch, W. (1992) *Nucleic Acids Res.* **20,** 1717–1723.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Le Fur, N., Kelsall, S. R., Silvers, W. K., and Mintz, B. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5332–5337.
15. Brichard, V., Van Pel, A., Wölfel, T., Wölfel, C., De Plaen, E., Lethé, B., Coulie, P., and Boon, T. (1993) *J. Exp. Med.* **178,** 489–495.
16. Robbins, P. F., and Kawakami, Y. (1996) *Curr. Opin. Immunol.* **8,** 628–636.
17. Tacke, R., Chen, Y., and Manley, J. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1148–1153.
18. Chandler, S. D., Mayeda, A., Yeakley, J. M., Krainer, A. R., and Fu, X.-D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3596–3601.
19. Sterner, D. A., Carlo, T., and Berget, S. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 15081–15085.