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# Short Communication

## Melanocyte Lineage-Specific Antigens Recognized by Monoclonal Antibodies NKI-beteb, HMB-50, and HMB-45 are Encoded by a Single cDNA

Gosse J. Adema,\* Annemiek J. de Boer,\*  
Rianne van 't Hullenaar,<sup>†</sup> Marylène Denijn,<sup>†</sup>  
Dirk J. Ruiter,<sup>†</sup> Arthur M. Vogel,<sup>‡</sup> and  
Carl G. Figdor\*

From the Division of Immunology,\* The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; the Department of Pathology,<sup>†</sup> University Hospital Nijmegen, The Netherlands; and the Department of Pathology,<sup>‡</sup> St. Louis University, St. Louis, Missouri

**The glycoproteins recognized by monoclonal antibody (MAb) NKI-beteb are among the best diagnostic markers for human melanoma. MAb NKI-beteb reacts with melanoma cells throughout tumor development and does not cross-react with other tumor or normal cells, except for cells of the melanocytic lineage. Two other melanocyte lineage-specific MAbs, HMB-50 and HMB-45, show a specificity and staining pattern strikingly similar to the ones observed for NKI-beteb. Herein, we demonstrate that all three MAbs recognize protein products encoded by a single cDNA. Expression of this cDNA in BLM cells results in immunoreactivity with all three MAbs. In addition, we demonstrate co-distribution of the RNA species detected by the cDNA with the proteins recognized by the MAbs in tissue sections. (Am J Pathol 1993, 143:1579-1585)**

The melanocyte lineage-specific antigens recognized by monoclonal antibody (MAb) NKI-beteb are among the best diagnostic markers for human melanoma available to date.<sup>1</sup> MAb NKI-beteb reacts with

melanoma cells throughout tumor development; it stains nevocellular nevi, primary cutaneous and uveal melanoma, melanoma metastases, and melanoma of soft tissues.<sup>1,2</sup> MAb NKI-beteb does not cross-react with other tumor cell types or normal cells, except for cells of the melanocytic lineage. The antigens recognized by MAb NKI-beteb are glycoproteins of 100 kd (gp100) and 7 kd (gp7), which are localized at the inside of premelanosomal vesicles.<sup>1</sup> Gp100 has also been detected in growth medium of melanoma cells cultured *in vitro*.<sup>1</sup>

Two other MAbs, HMB-45 and HMB-50, have been described that show a specificity and staining pattern very similar to the ones observed for MAb NKI-beteb.<sup>3,4</sup> MAb HMB-50 identifies a glycosylated, intracellular protein of 95 kd that is released into the growth medium by melanoma cells and neonatal melanocytes *in vitro*.<sup>4</sup> In addition, MAb HMB-50 immunoprecipitates a 10 kd protein from melanoma-cell extracts.<sup>4</sup> The antigen recognized by MAb HMB-45 localizes in premelanosomal vesicles<sup>5</sup> and has been suggested to have a molecular weight of 10 kd.<sup>6</sup> In contrast to MAb NKI-beteb, neither of these MAbs react with resting adult melanocytes.<sup>3,4</sup>

Despite their importance in diagnostic pathology of malignant melanoma, the primary structure of the melanocyte lineage-specific antigens recognized by each of these MAbs remains undefined. Herein,

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Address reprint requests to Dr. Gosse J. Adema, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. Current address of AMV: Cytolab., Seattle, WA 98107.

we report the isolation of a cDNA, which not only encodes the antigens recognized by MAb NKI-beteb, but also those recognized by MAbs HMB-45 and HMB-50.

## **Materials and Methods**

### *Cells and Tissues*

The melanoma cell lines MEL-2a, MEL-1, M14, MEL-57, MEWO, and BLM were described previously.<sup>1,4,7-10</sup> Isolation of normal human melanocytes from breast or foreskin was performed by the method of Eisinger and Marko<sup>11</sup> with modifications as described previously.<sup>12,13</sup> Normal fetal epidermal tissue (approximately 15 weeks) and three primary cutaneous melanomas were obtained after surgery and immediately frozen in liquid nitrogen until use.

### *cDNA Library Construction, Screening, and Subcloning*

A  $\lambda$ -gt11 cDNA expression library was constructed from poly(A)<sup>+</sup> RNA isolated from the human melanoma cell line MEL-1 and screened with the rabbit polyclonal antiserum against the 95 kd glycoprotein<sup>4</sup> using standard techniques.<sup>14</sup> The cDNA insert of a positive clone,  $\lambda$ -gp100-c1, was removed as an *EcoRI* fragment, blunt-ended by filling in the ends with Klenow DNA polymerase and then ligated in the *Sma* I site of pRSV328A<sup>15</sup> in either the coding (pRSVgp100+) or the noncoding (pRSVgp100-) orientation.

### *RNA Isolation and Northern Blot Analysis*

Total RNA was prepared by the guanidine thiocyanate procedure and centrifugation through a cushion of cesium chloride.<sup>16</sup> RNA samples (5 to 30  $\mu$ g) were size fractionated on a formaldehyde-agarose gel, transferred onto Hybond-N membranes (Amersham), and hybridized to  $\alpha$ -[<sup>32</sup>P]-dATP-labeled probes (random primed DNA labeling kit, Boehringer Mannheim, Mannheim, Germany). As probes we have used the 5' *EcoRI-Nco* I fragment ( $\pm$ 550 bp) or the 3' *Bst*XI-*EcoRI* fragment ( $\pm$ 450 bp) of gp100-c1 cDNA.

### *Transfection and Immunofluorescence*

BLM cells were transfected with 20  $\mu$ g of DNA by the calcium phosphate co-precipitation procedure<sup>17</sup>

using calcium phosphate transfection systems (Bethesda Research Laboratories, Bethesda, MD). Five hours after the addition of the precipitate cells were shocked with either dimethylsulfoxide (10% in medium for 90 seconds) or glycerol (10% in medium for 3 minutes). Forty-eight hours after transfection cells were prepared for immunofluorescence (fluorescein isothiocyanate) as described previously.<sup>1</sup> MAbs NKI-beteb and HMB-50 were described previously.<sup>1,4</sup> MAb HMB-45 was purchased from Enzo Biochem. Preparations were examined on a confocal laser scanning microscope at 488 nm (Bio-Rad MRC 600).

### *Immunocytochemistry and In Situ Hybridization*

For immunocytochemistry, serial cryosections (5  $\mu$ ) were fixed in acetone (10 minutes) then rehydrated and incubated with MAbs NKI-beteb (Zymed Laboratories, 1:150), HMB-45 (ENZO Biochem, 1:80), or HMB-50 ascites (1:1000) for 1 hour at 20°C. Subsequently, sections were washed then incubated with biotinylated horse anti-mouse antiserum (Vector, 1:200) for 30 minutes at 37°C then washed again and incubated with an avidin-biotin-peroxidase complex (Vector, 1:100) for 30 minutes at 37°C. The immunohistochemical reaction was visualized with amino-ethyl-carbazole/H<sub>2</sub>O<sub>2</sub>. Finally, the sections were counterstained with hematein and embedded in Kaiser's glycerin. Cryosections not incubated with a primary MAb served as a control.

RNA *in situ* hybridization was performed on cryosections (8  $\mu$ ) adjacent to those used for immunocytochemistry as described earlier.<sup>18,19</sup> As a probe we used a  $\alpha$ -[<sup>35</sup>S]-dCTP random primed labeled *EcoRI-Nco* I fragment ( $\pm$  550 bp) of gp100-c1 cDNA. As a control, cryosections were hybridized to  $\alpha$ -[<sup>35</sup>S] random primed labeled phage  $\lambda$ -DNA.

## **Results**

Based on the immunohistochemical and biochemical data described above, we assumed that the gp100 antigen recognized by MAb NKI-beteb and the 95 kd glycoprotein recognized by MAb HMB-50 share a similar, if not identical protein backbone. Therefore, we used a rabbit polyclonal antiserum against the 95 kd glycoprotein to screen a  $\lambda$ -gt11 cDNA expression library prepared from poly(A)<sup>+</sup> RNA isolated from human melanoma cells.<sup>4</sup> A positive clone,  $\lambda$ -gp100-c1, containing a cDNA insert of approximately 2.2 kb was obtained and used for

further analysis. To detect the RNA species from which the cDNA is derived, Northern blot analysis was performed using either the 5' part ( $\pm$  550 bp) or the 3' part ( $\pm$  450 bp) of gp100-c1 cDNA as a probe. Both probes specifically detected RNA species of 2.5 kb, 4.2 kb, and a high molecular weight RNA species ( $>$ 15 kb) in total RNA isolated from adult and neonatal melanocytes as well as in total RNA isolated from four of five human melanoma cells (Figure 1 and results not shown). The same RNA species were detected in poly(A)<sup>+</sup> RNA isolated from human melanoma cells (not shown). Note that no hybridizing RNA species were detected in RNA from BLM melanoma cells, consistent with the absence of reactivity with the MAbs (see below).

*GP100-C1 cDNA Encodes a Protein that is Recognized by MAbs NKI-beteb, HMB-50, and HMB-45*

To determine whether gp100-c1 cDNA encodes the antigen recognized by MAbs NKI-beteb and/or HMB-50, we cloned the cDNA into the eukaryotic expression vector pRSV328A<sup>15</sup> in either the coding (pRSVgp100+) or the noncoding (pRSVgp100-) orientation and transfected these constructs in BLM melanoma cells. BLM cells were selected for the

transfection experiments, because they do not react with MAbs NKI-beteb, HMB-50, or HMB-45 nor express the RNA species detected by the gp100-c1 cDNA probe (see above). As shown in Figure 2 B and H, BLM cells expressing the transcription unit containing the cDNA in the coding orientation reacted with MAb NKI-beteb and MAb HMB-50. The granular, cytoplasmic staining pattern observed with MAbs NKI-beteb and HMB-50 in the transfected cells is very similar to the one observed in MEWO melanoma cells, which express the antigens endogenously (Figure 2 A and G). No immunoreactivity to either MAb was observed when BLM cells were transfected with the construct containing the cDNA in the noncoding orientation (Figure 2 C and I).

The similar reactivity of MAbs NKI-beteb and HMB-45 to cells of the melanocytic lineage led us to investigate whether MAb HMB-45 reacts with the proteins encoded by gp100-c1 cDNA. As shown in Figure 2 E and F, only BLM cells transfected with pRSVgp100+ reacted with MAb HMB-45. The staining pattern observed with MAb HMB-45 in transfected BLM cells was similar to the one observed with MAbs NKI-beteb and HMB-50 and mimicked the one of HMB-45-stained MEWO melanoma cells (Figure 2 B, D, E, and H). We conclude from these data that a single cDNA encodes the melanocyte lineage-specific antigens recognized by MAbs NKI-beteb, HMB-50, and HMB-45.

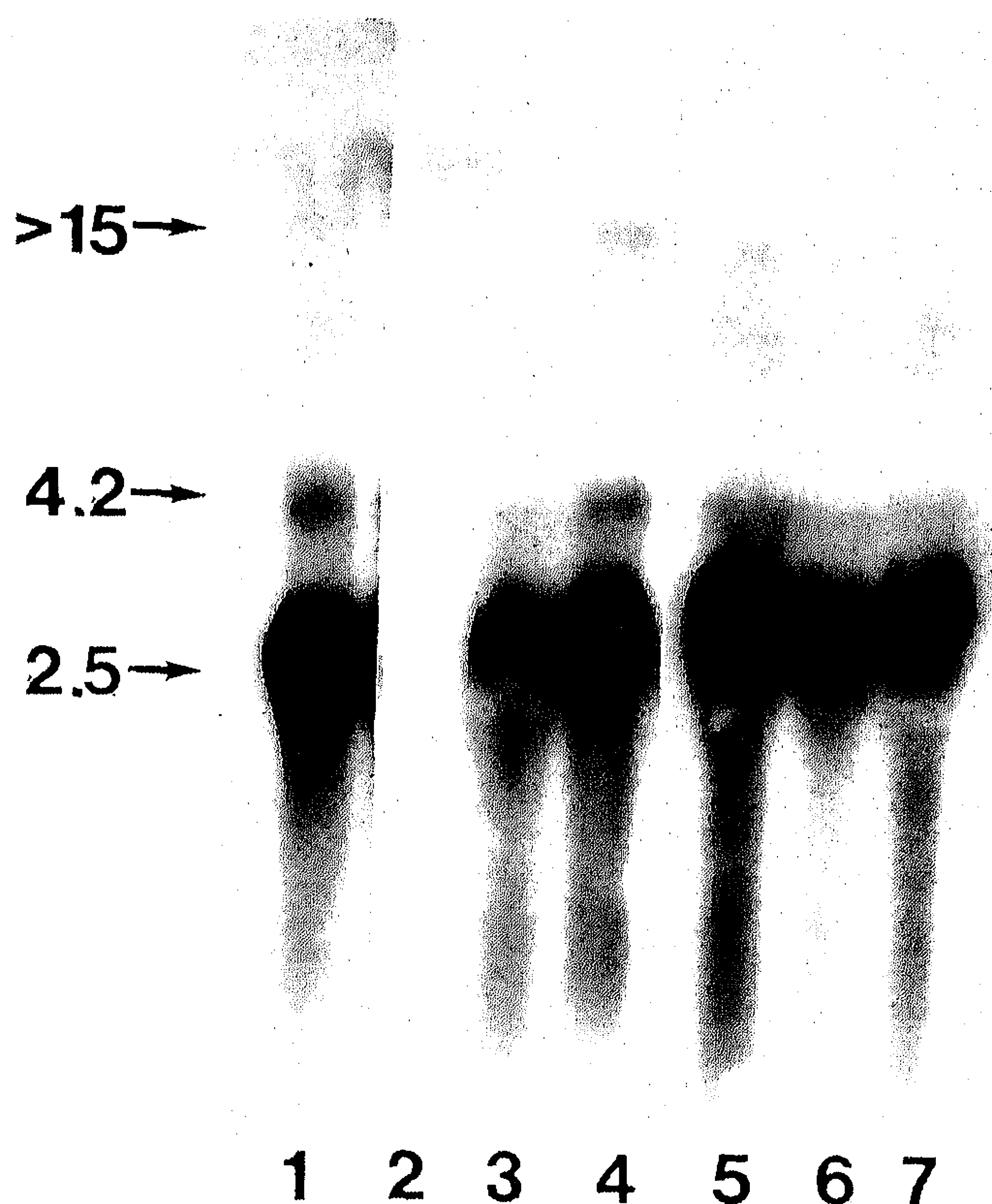


Figure 1. RNA species detected by gp100-c1 cDNA in cells of the melanocytic lineage. Northern blot analysis of total RNA isolated from adult (lane 1) and neonatal (lane 4) melanocytes as well as from five different melanoma cell lines (BLM, lane 2, MEWO, lane 3, MEL-2a, lane 5, MEL-57, lane 6, and M14, lane 7). The Northern blot was hybridized to the 5' EcoRI-Nco I fragment of gp100-c1 cDNA. The size (kb) and position of the hybridizing RNA species are indicated by an arrow. The same results were obtained using the 3' Bst XI-EcoRI fragment of gp100-c1 cDNA as a probe (not shown).

*In Situ Detection of GP100 RNA and Protein in Malignant Melanomas and Fetal Skin*

Because of their specificity for cells of the melanocytic lineage MAbs NKI-beteb, HMB-45, and HMB-50 are important tools in the diagnosis of malignant melanoma. To compare the distribution of the proteins recognized by these MAbs with the expression pattern of the RNA species detected by gp100-c1 cDNA, we performed *in situ* hybridization in combination with immunocytochemistry on sequential cryosections of primary melanomas and fetal skin. In two primary melanomas co-distribution of gp100 RNA and the proteins recognized by each of the MAbs was observed (Figure 3). Beside melanocytes and/or melanoma cells, no other cells reacted with the cDNA probe or the MAbs. One other primary melanoma we selected as a control did not react with any of the MAbs nor with the cDNA probe (not shown). This result demonstrates that for the tumor biopsies the presence or absence of reactivity with all three MAbs and

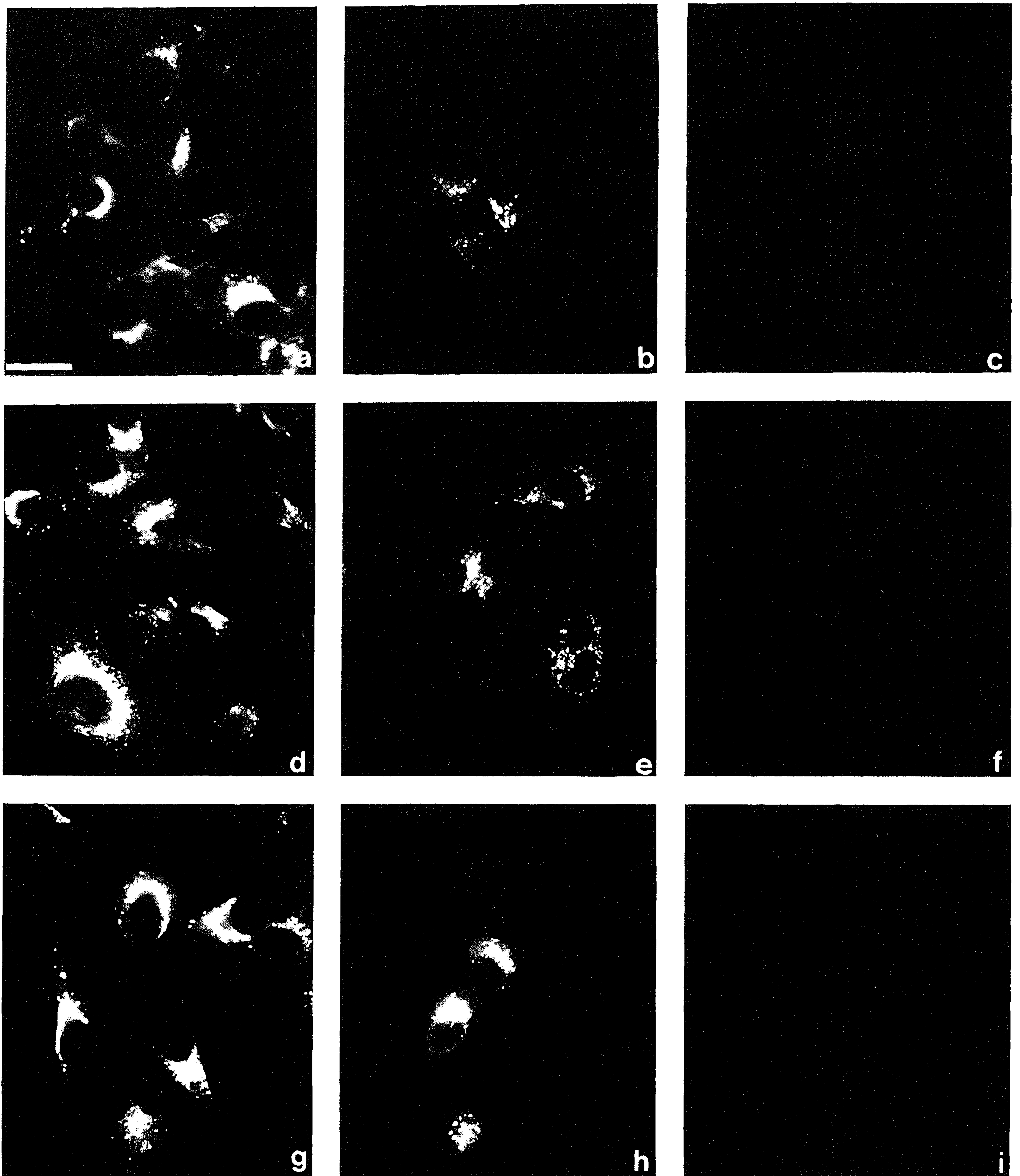


Figure 2. Expression of the cDNA clone in BLM cells transfers immunoreactivity to MAbs NKI-beteb, HMB-45, and HMB-50. BLM cells transfected with either *pRSVgp100+* (B, E, H) or *pRSVgp100-* (C, F, I) and MEWO cells (A, D, G) were analyzed for reactivity to MAbs NKI-beteb (A to C), HMB-45 (D to F), or HMB-50 (G to I). Indirect immunofluorescence (fluorescein isothiocyanate) was examined by confocal laser scanning microscopy. Magnification is indicated by a size bar (25  $\mu$ ) in A.

with gp100-c1 cDNA are mutually exclusive. Co-distribution of RNA and protein in fetal skin was most pronounced in melanocytes present in hair follicles (not shown). Hair follicles cut at a position devoid of melanocytes did not react with the cDNA probe or the

MAbs (not shown). Taken together, these results clearly demonstrate specific co-distribution of RNA species detected by gp100-c1 cDNA with the proteins recognized by all three MAbs in cells of the melanocytic lineage.

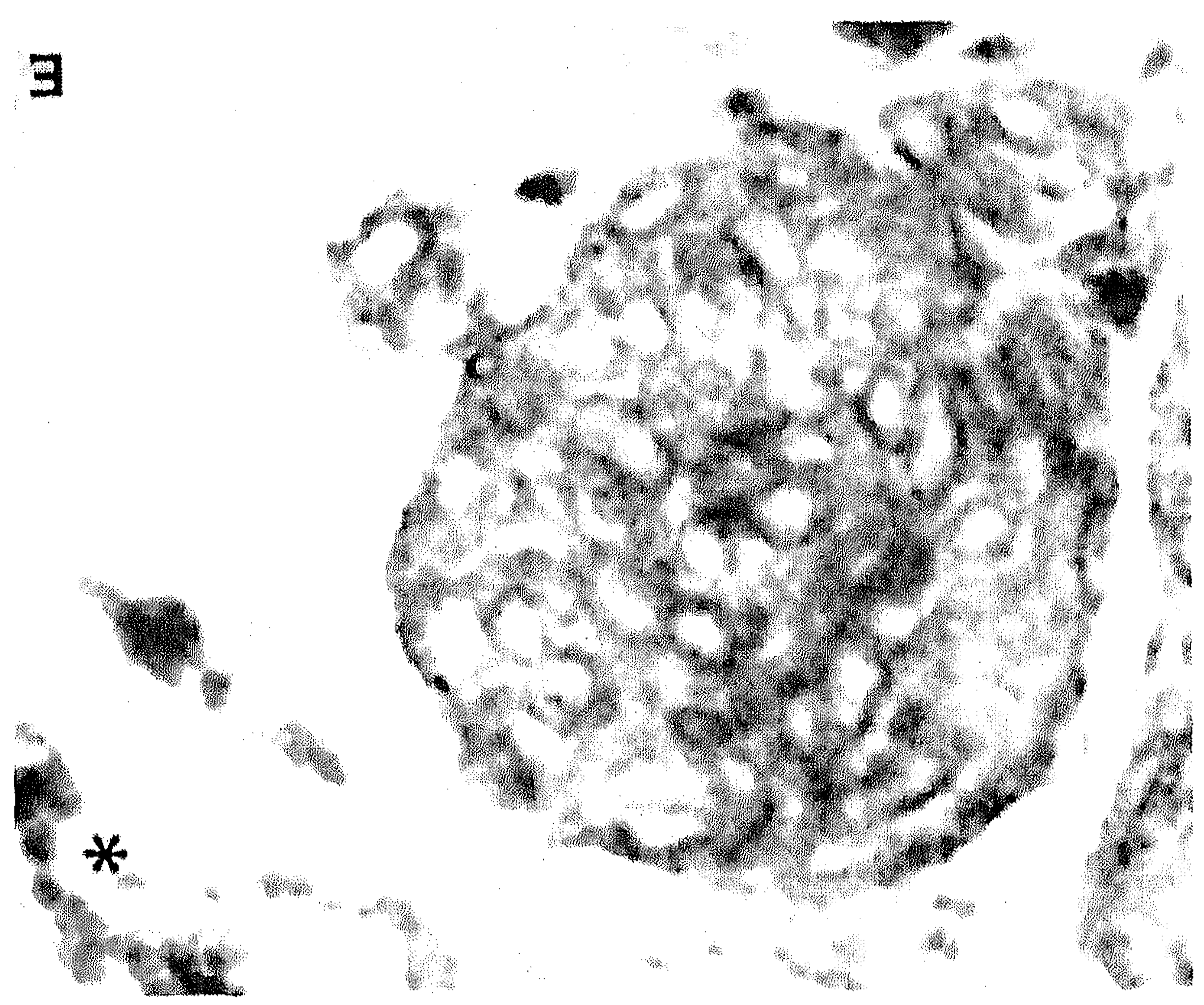
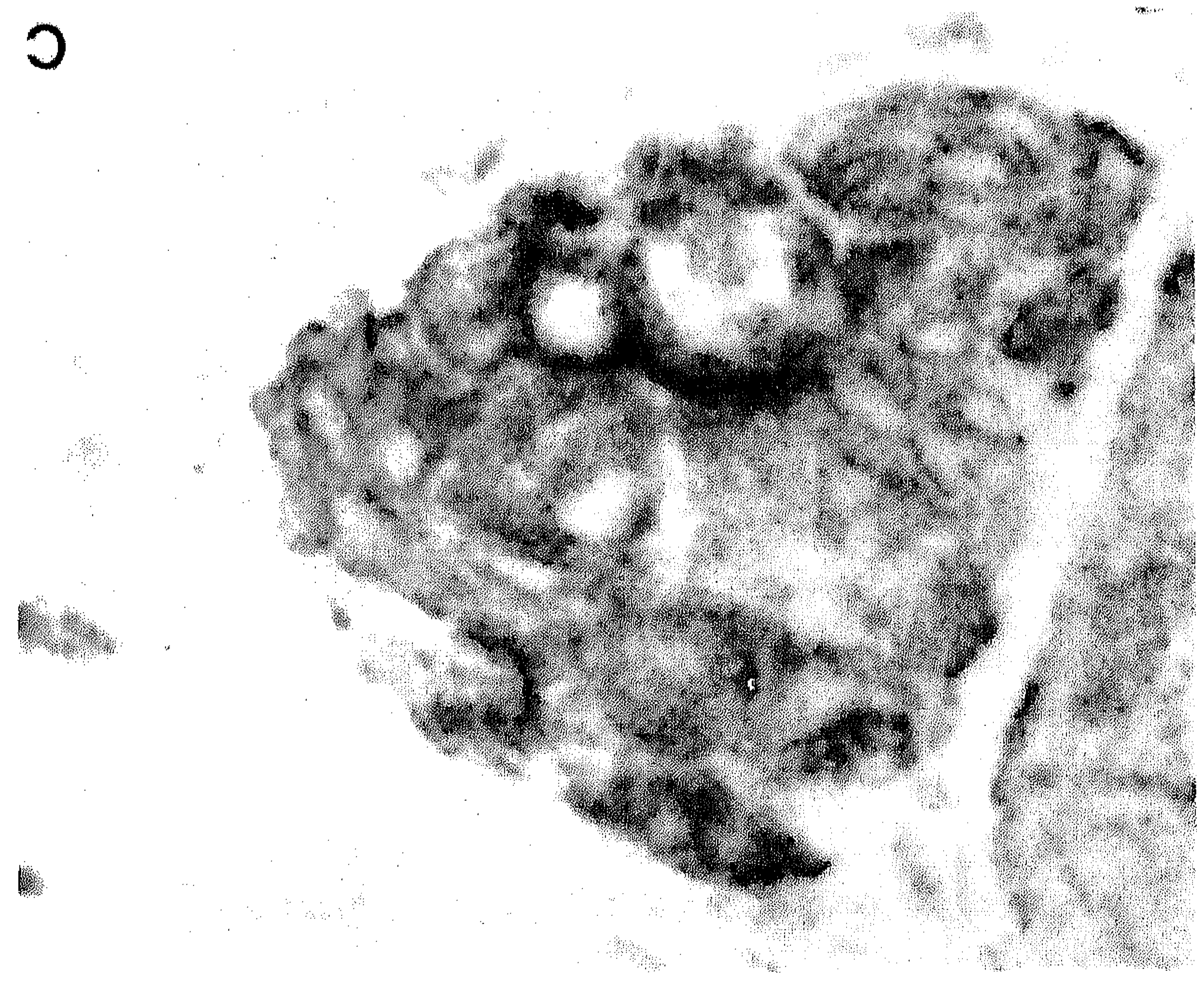
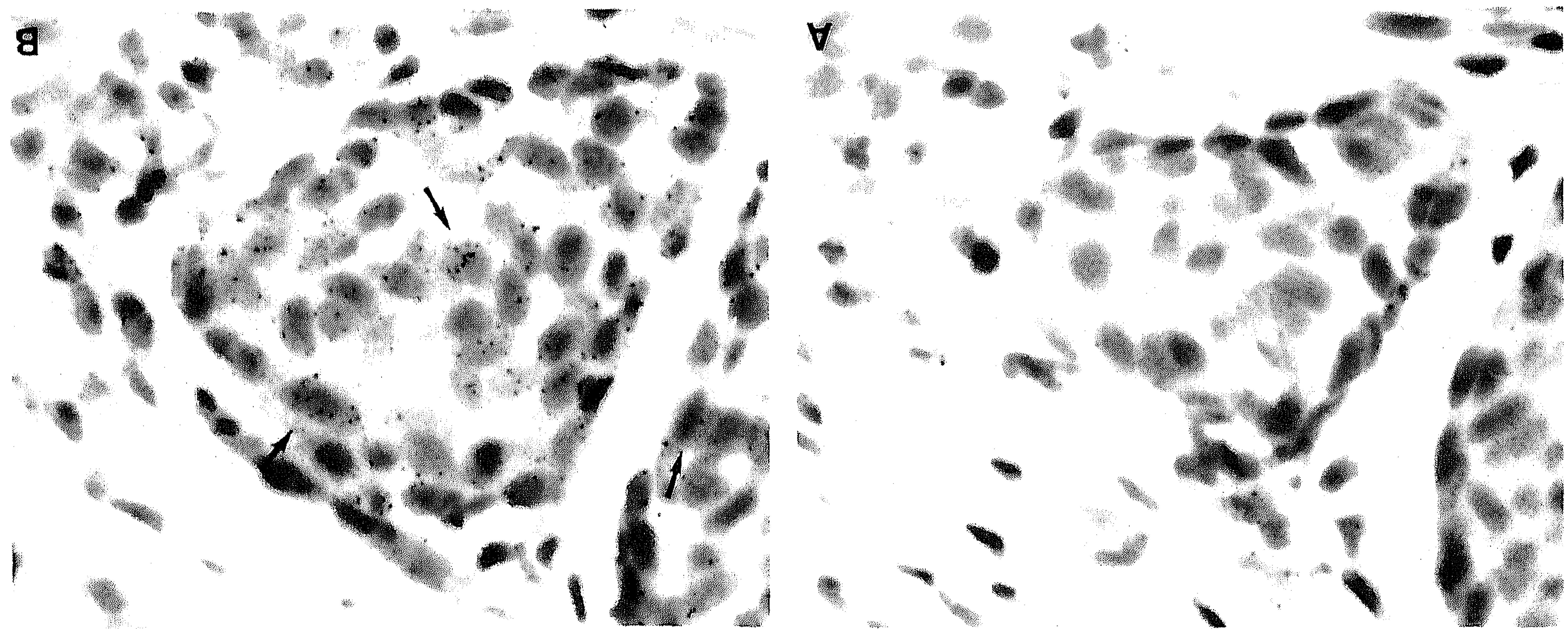


Figure 3. Detection of gp100 RNA and protein in a primary melanoma. Sequential cryosections were hybridized with <sup>35</sup>S-labeled phage λ-DNA (A), the <sup>35</sup>S-labeled 5' part of gp100-c1 cDNA (B), or stained with Mab NK1-beta6 (C), Mab HA1B-45 (D), or Mab HA1B-50 (E) using the avidin-biotin-peroxidase method. Cryosections not incubated with a primary Mab served as a control (not shown). A number of gp100 RNA containing melanoma cells are indicated by arrows. Melanophages are indicated by an asterisk. Magnification ×40.

HMB-45, and HMB-50.<sup>1,3,4</sup> Expression of this cDNA in BLM cells, which are negative for all three Mabs and do not express RNA species hybridizing to the cDNA probe, results in immunoreactivity to Mabs NK1-beta6 and HMB-50. Interestingly, we found that

Herein, we show that gp100-c1 cDNA encodes the melanocyte lineage-specific antigens recognized by three previously described Mabs, NK1-beta6,

*Discussion*

MAB HMB-45,<sup>3</sup> which is widely used in diagnostic pathology because of its specificity and sensitivity on paraffin-embedded tissue sections, also reacts with BLM cells expressing gp100-c1 cDNA. This result is consistent with previous data suggesting that MABs NKI-beteb and HMB-45 recognize different epitopes on the same antigen.<sup>1</sup>

An RNA species of 2.5 kb is the major RNA product detected by the cDNA probe in melanoma cells as well as in adult and neonatal skin melanocytes. In these cells, additional RNA species of 4.2 and >15 kb were also discerned. The finding that the 5' part and the 3' part of gp100-c1 cDNA detect the same RNA species indicates that the cDNA isolated is derived from a single RNA species. The size of the major polyadenylated RNA species detected (2.5 kb) is also consistent with the size of gp100-c1 cDNA (2.2 kb). Using *in situ* hybridization in combination with immunocytochemistry on sequential sections of primary melanomas and fetal skin, we demonstrate co-distribution of gp100 RNA with proteins reacting to all three MABs in cells of the melanocytic lineage. These findings provide additional evidence that gp100-c1 cDNA encodes the antigens recognized by all three MABs. The notion that the melanocyte lineage-specific antigens recognized by MABs NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA is also fully consistent with the finding that proteins of 100 and 10 kd are immunoprecipitated by MABs NKI-beteb and HMB-50 from nonpigmented COS cells expressing gp100-c1 cDNA (G.J. A. et al, manuscript submitted).

Comparison of the reported reactivity of MABs NKI-beteb, HMB-45, and HMB-50 to subpopulations of melanocytic cells reveals that all three MABs react with melanoma cells and neonatal and fetal melanocytes, but that only MAB NKI-beteb recognizes resting adult melanocytes in skin.<sup>1,3,4</sup> This observation led to the hypothesis that MABs HMB-50 and HMB-45 detect oncofetal proteins.<sup>3,4</sup> However, the finding that all three MABs recognize the protein encoded by a single cDNA argues against this hypothesis and suggests a role for posttranslational protein modification. Aberrations in posttranslational modification (ie, glycosylation) of proteins are known to frequently occur in tumor cells, including melanoma cells.<sup>20</sup> Actually, differences in the posttranslational modification of the antigens detected by HMB-50 in melanoma cells compared with neonatal foreskin melanocytes have been reported.<sup>4</sup> In addition, results of a recent study suggest that the epitope recognized by MAB

HMB-45 is dependent on the presence of sialated glycoconjugates.<sup>21</sup> Furthermore, we found that cultured adult melanocytes strongly react with all three MABs (not shown).<sup>13</sup> This latter result is consistent with previous data showing that beside neoplastic transformation, melanocyte stimulation also results in immunoreactivity to MAB HMB-45.<sup>22,23</sup> Collectively, these data implicate that MABs HMB-45 and HMB-50 recognize posttranslational modifications of a constitutively expressed melanocyte-specific protein, not a *de novo* synthesized oncofetal protein.

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