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Expression of c-jun, jun-B, and c-fos Proto-Oncogenes in Human Primary Melanocytes and Metastatic Melanomas

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Analysis of the regulation of c-jun, jun-B, and c-fos RNA transcript expression was performed in human primary melanocytes and metastatic melanoma cell strains. The medium requirements for human melanocyte in vitro growth are phorbol esters, agents that elevate intracellular cAMP levels, hormones, and growth factors. Cellular jun, jun-B, and c-fos gene expression are known to be affected by growth promoting agents. In primary melanocytes, the expression of c-jun, jun-B, and c-fos RNA transcripts was dependent on the growth-promoting agents present in the medium. Uniformly high c-jun, jun-B, and c-fos RNA transcript levels were observed in melanocytes cultivated in complete medium. Higher levels of c-jun RNA transcripts and low levels of c-fos RNA transcripts were observed in melanocytes cultivated in plain medium. In contrast, a range of c-jun, jun-B, and c-fos RNA transcript levels was detected in metastatic melanoma cell strains cultivated in medium with or without serum. In general, an increase in jun-B and c-fos RNA transcript expression and a decrease in c-jun RNA transcript expression was observed in metastatic melanomas compared to neonatal melanocytes. These data suggest a potential role for c-jun, jun-B, and c-fos genes in the transformation of melanocytes to malignant melanoma. *J Invest Dermatol 97:349–353, 1991*

Human melanomas are highly resistant to radiation and chemotherapy; the only effective treatment is early surgical excision. Information on the factors regulating the formation of melanoma could lead to new tumor therapy. Within the past decade, it has become possible to culture human primary melanocytes from neonatal foreskin epidermis. Melanocytes can be cultured for a limited time and require media containing phorbol esters, serum, hormones, agents that elevate cAMP levels, and bovine pituitary extracts or basic fibroblast growth factor [1,2]. These growth-inducing agents are known to affect the expression of various genes.

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), affects the expression of many genes (for review, see [3]). One of the transcription factors involved in affecting the expression levels of TPA-inducible genes is the c-jun protein. The c-jun gene has been shown to belong to a gene family; two other members include jun-B and jun-D [4]. The oncogene jun-B has been shown to inhibit the transforming and transactivating activities of the c-jun oncogene [5]. Cellular jun protein is one of several polypeptides that form a complex called AP-1, a heterodimer that may consist of c-fos and c-jun proteins. The AP-1 complex recognizes a specific DNA sequence (TPA responsive element) that mediates the transcriptional response to phorbol esters [23]. Some of the TPA-inducible genes are oncogenes (c-fos, c-myc, and c-sis), proteases (collagenase, and stromelysin), and lymphokines (interleukin 1β and 2) (for review, see [3,33,34]). Thus an induction in the protein level of the AP-1 complex could induce the expression of various genes that assist in the transformation of human melanocytes.

Transcriptional activation of the c-fos oncogene by various agents has been investigated by many researchers. Cellular fos RNA transcript levels are induced during stimulation of cells with growth factors, mitogens, and DNA damage (for review, see [6,35]). Cellular fos RNA transcript expression has been studied in a murine, immortalized, nontumorigenic melanocyte cell line. Hart et al [7] observed an increase in c-fos RNA transcripts following treatment of murine melanocytes with N^6, 2′-O-dibutyryladenosine, 3′-5′ cyclic monophosphate (dbcAMP). Investigators have also studied the inhibition of c-fos RNA transcript expression by antisense fos gene expression vectors. Both the proliferation of dividing cells [8], and the proliferation of quiescent cells stimulated with mitogens [9] has been blocked by antisense fos. Cellular fos gene is an oncogene whose elevated expression level can induce tumors in vivo [10] and transformation of cells in vitro [11].

Only limited information is available on the molecular biology of primary melanocytes and metastatic melanoma (for recent reviews, see [12,13]). We report our investigation of the RNA expression of the transcription factors c-jun, jun-B, and c-fos oncogenes in the proliferation response of melanocytes and metastatic melanoma cell strains. We demonstrate that c-jun gene expression was repressed and jun-B and c-fos genes expression was inducible in human melanocytes. These data suggest a potential role for these genes in the transformation of human melanocytes to melanoma.

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Abbreviations:

Ca^{++}: calcium ions
cAMP: adenosine 3′,5′-cyclic monophosphate
dbcAMP: N^6-2′-O-dibutyryladenosine, 3′,5′-cyclic monophosphate
RNA: ribonucleic acid
SDS: sodium dodecyl sulfate
SSC: saline sodium citrate

MATERIALS AND METHODS

Culture of Melanocytes The method used is a combination of the procedures developed by Eisinger and Marko [14] and Halaban and Alfano [15]. Foreskin samples were collected from newborn infants, and the melanocytes isolated and transferred to a T-75 flask. Primary newborn melanocytes were cultivated in MCDB 153 medium (Irvine Scientific, Irvine, CA) as described by Halaban et al [1] and modified by J. Jambrosic et al [16]. Fibroblast contamination was suppressed by adding geneticin (250 μg/ml) to the growing medium for 2 d. Melanoma cell strains (c81-46a, c81-46c, c81-61, and c83-2cy) were cultured in F-10 with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) [17–19]. The passage number for the various cell strains used in these experiments was less than eight. Cell strains c81-46a and c81-46c are from two different biopsy sites isolated from the same patient and are slow growing, but are free of mycoplasma as determined using a Gen-probe kit (Fisher, Pittsburgh, PA). Cell strain c81-61 is an intermediate growing cell line. Cell strain c83-2cy was isolated from a metastatic melanoma that was passed once through a nude mouse and grows rapidly. Viable cell counts were determined by trypan blue exclusion.

Assessment of Cell Proliferation Melanocytes were seeded into either six-well (8,000 cells) or 96-well (2,700 cells) plates (Falcon, Lincolin Park, NJ) and allowed to grow to 50% confluency. Wells were rinsed and medium replaced with the various growth media. After various times, melanocytes were isolated and the cell number determined. Cell proliferation was also determined by amount of incorporated radioactivity. DNA synthesis was measured by labeling with [methyl-^{3}H]-thymidine (2.5 μCi/ml, 20 Ci/mmol, Dupont-New England Nuclear, Boston, MA) added to the medium during the last 24 h of the treatment period. [^{3}H]-thymidine incorporation into insoluble material was isolated using a Ph.D. cell harvestor (Cambridge Research Inc., Valley Stream, NY). Amount of radioactivity incorporated was determined by liquid scintillation counting (LS5000TD, Beckman, Fullerton, CA) with an efficiency of 62.7%.

Isolation of RNA and Northern Blot Analysis The procedure is a modification of that described by Chirgwin et al [20]. Two to six T-175 flasks were used per growth condition, and the cells were allowed to grow to 70–80% confluency. The cells were rinsed with prewarmed medium an various prewarmed growth media were added to each flask. The cells were cultivated for either 48 or 72 h in the various growth media. The cells were pelleted and lysed using a 4-M guanidine isothiocyanate–1% Sarkosyl solution. The sheared homogenate was layered on top of a 5.7–M cesium chloride cushion and centrifuged at 55000 rpm for 3 h. The RNA pellet was resuspended in TE with 0.1% SDS, extracted, ethanol precipitated, and resuspended in sterile water. The Northern blot was prepared as described by Fourney et al [21]. Ten μg of total RNA per sample were electrophoresed on a denaturing formaldehyde agarose gel, transferred by capillary action overnight to a nylon filter (Nytran, Schleicher and Schuell, Keene, NH), and prehybridized for 4 h at 42°. The probe was labeled by the method of Feinsberg and Vogelstein [22] using a random priming kit (Promega, Madison, WI) and added with fresh hybridization solution as described by manufactors' procedures to the filter. Hybridization was done overnight and two to three stringent washouts were done at 50° with 0.1% SDS and 0.1 × SSC. The filter was exposed for 2 hours to overnight at −80° to Kodak X-OMAT film.

Densitometry For quantification of individual RNA, films were exposed for time periods during which band intensity was linear with respect to time. Films were then scanned with a laser densitometer (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ) and RNA transcript abundance was determined from the area of the peak corresponding to each RNA transcript. RNA standards were used to determine RNA sizes (0.24–9.5-kb RNA ladder, BRL, Gaithersburg, MD).

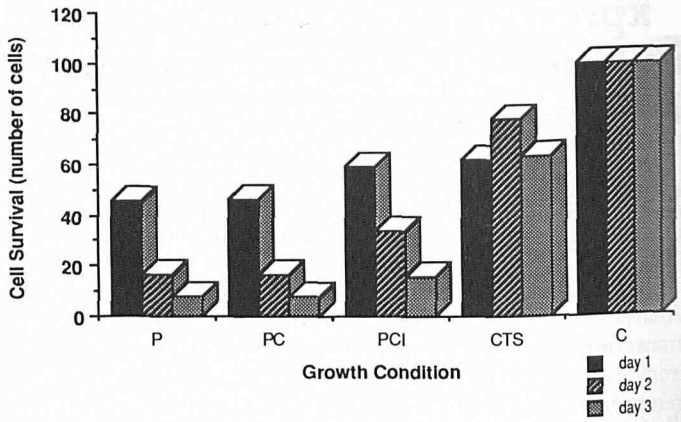

Figure 1. *Effects of growth media on cell proliferation in human primary melanocytes.* Subconfluent cultures were switched to each medium for various times and cells counted. Data are means of two to three independent experiments with three replicate 9.6-cm^2 cultures/growth medium/experiment. P, MCDB 153 medium only; PC, plain with calcium ions (2.0 mM); PCI, plain with calcium ions (2.0 mM) and insulin (5 μg/ml); CTS, complete medium without TPA (10 ng/ml) and serum (2%); and C, complete medium.

Probes The c-jun data were obtained with a 1.2-kb SalI-HpaI fragment from the plasmid pHJ [23]. A jun-B 1.1-kb ScaI fragment from the plasmid 465.2 was used to probe for jun-B RNA transcript expression [24]. A 1.8-kb XhoI-EcoRI fragment from the plasmid BK28 [25] was used to probe for c-fos expression. Standardization for the amount of RNA loaded was obtained by using a 5.6-kb EcoRI fragment of an 18S DNA from the plasmid, pB [26]. Purified DNA inserts for the various genes were used in all studies.

RESULTS

Growth Agent Requirements Early-passage neonatal foreskin melanocytes ($p < 6$) were used to determine the minimum growth-agent requirements to induce melanocytes to a quiescent state within 48 h [1,16,27]. The effects of different media on the growth of human melanocytes are presented in Fig 1. Melanocytes cultivated in complete media had a population doubling time of 1.7 d. Melanocytes proliferated in complete media minus TPA and serum with a population doubling time of 4 d. No increase in cell number was observed in the plain media (medium MCDB 153 only) or plain media supplemented with 2 mM calcium ions (Ca^{++}) or Ca^{++} and insulin. DNA synthesis was measured after cultivation of primary melanocytes in various growth conditions for 48 h (Fig 2). Reduced [^{3}H]-thymidine incorporation levels were observed for primary melanocytes cultivated in plain medium or supplemented with Ca^{++} or Ca^{++} and insulin (< 10%) compared to primary melanocytes cultivated in complete medium. Cultivation of primary melanocytes in complete medium without TPA and serum was one third the level of [^{3}H]-thymidine incorporation of primary melanocytes cultivated in complete medium.

Northern Analysis Using the various growth conditions, total RNA was isolated and probed for expression of c-jun, jun-B, and c-fos RNA transcripts. The densitometric scans of the c-jun, jun-B, and c-fos data (Figs 3, 4, and 5) are summarized in Table I. Northern analysis detected two bands (2.7 kb and 3.4 kb) for c-jun RNA transcript expression in human primary melanocytes and metastatic melanomas (Fig 3). The expression of the two transcripts was different in melanocytes and melanomas. In melanocytes cultivated in medium without any growth factor supplements (plain medium) for 72 h, only a faint level of the 2.7-kb c-jun RNA transcripts was detected. When RNA was extracted from primary melanocytes cultivated in the growth conditions for 48 h, high levels of the

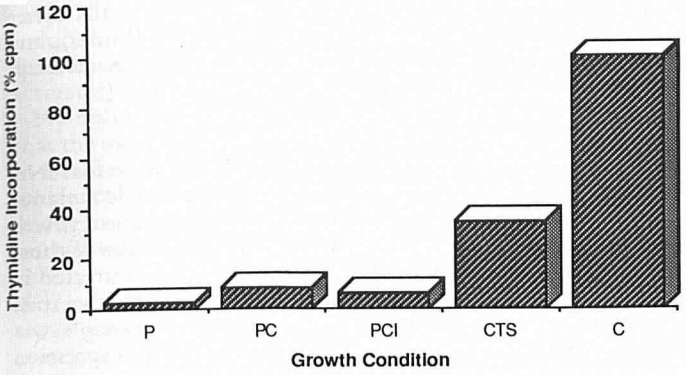

Figure 2. Effects of growth media on DNA synthesis in human primary melanocytes. Subconfluent cultures were switched to each media for various times and pulsed with [^{3}H]-thymidine as described in *Materials and Methods*. Data are means ± SE of six replicate cultures/condition. Growth conditions are as described in Fig 1.

2.7-kb c-jun RNA transcript were detected. Melanocytes cultivated in plain medium or supplemented with Ca^{++} ions or Ca^{++} and insulin were observed to express a high level of the 2.7-kb c-jun RNA transcript. Cultivation of melanocytes in complete medium without TPA and serum or complete medium led to a decrease (0.28 times and 0.26 times, respectively) in c-jun RNA transcript levels compared to the previously mentioned growth conditions.

There were different levels of the 2.7-kb c-jun RNA transcript expressed in the four melanoma cell strains cultivated in medium with or without serum. Cell strain c81-46a expressed c-jun RNA transcripts at the same level in medium with or without serum. Expression of the 2.7-kb RNA transcript was induced (5.77 times) in c81-61 cells when cultivated in medium with serum compared to medium without serum. In contrast, c-jun RNA transcript levels were reduced in cell strains c81-46c and c83-2cy. Cell strain c81-46c expressed c-jun RNA transcripts, which were reduced (0.32 times) when cultivated in medium with serum compared to medium without serum. Cellular jun RNA transcripts were reduced (0.69 times) when c83-2cy cells were cultivated in medium in serum compared to medium without serum.

Expression of the 3.4-kb c-jun RNA transcript was different from the expression of the 2.7-kb c-jun RNA transcript. As the melanocytes were cultivated in more complete medium, there was a decrease in the expression of the 3.4-kb c-jun RNA transcripts; the transcript was faint when melanocytes were cultivated in complete medium. In the melanomas cultivated in medium with or without

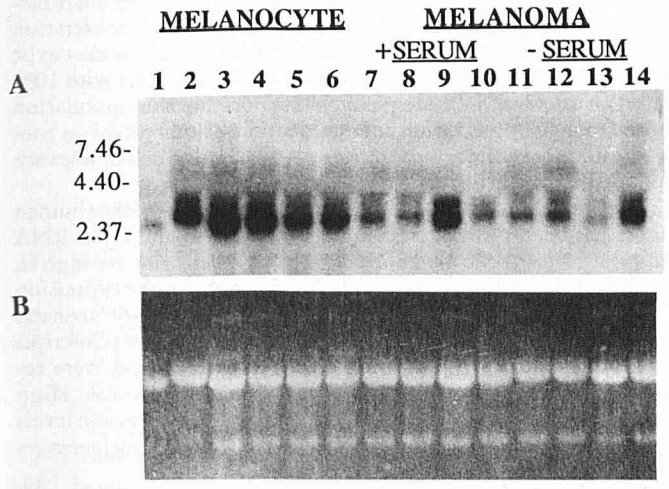

Figure 3. Expression of c-jun RNA transcripts in various growth medium from primary melanocytes and metastatic melanomas. Cells were cultivated and treated for 48 to 72 h in the various conditions described in *Materials and Methods*. Ten micrograms of total RNA was hybridized with ^{32}P-labeled restriction fragments with either c-jun *(A)* or ethidium bromide staining of gel *(B)*. *Lane 1,* P for 3 d; *lane 2,* P; *lane 3,* PC; *lane 4,* PCI; *lane 5,* CTS; *lane 6,* C; *lanes 7 and 11,* c81-46a; *lanes 8 and 12,* c81-46c; *lanes 9 and 13,* c81-61; *lanes 10 and 14,* c83-2cy. Growth conditions are as described in Fig 1. Numbers on the left show the positions of migration by RNA standards with indicated kilobase length.

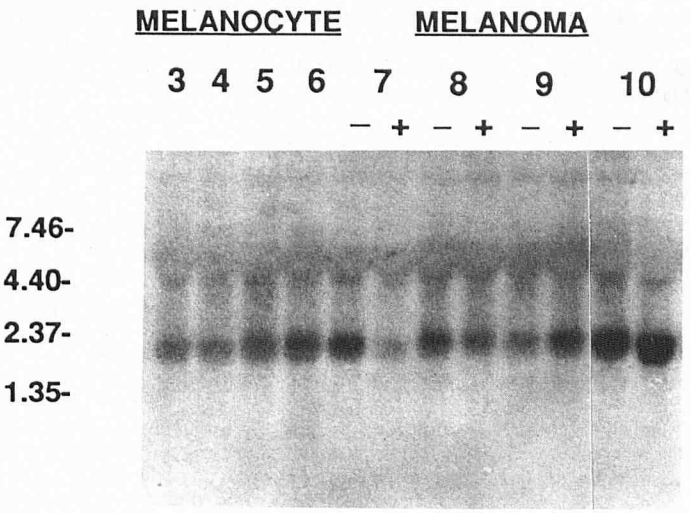

Figure 4. Expression of jun-B RNA transcripts in various growth medium from primary melanocytes and metastatic melanomas. Cells were cultivated and treated for 48 h in the various conditions described in *Materials and Methods*. Ten micrograms of total RNA was hybridized with ^{32}P-labeled restriction fragments with jun-B probe. Lane conditions, growth conditions, and RNA standards are as described for Fig 3.

Expression of the 3.4-kb c-jun RNA transcript was different from the expression of the 2.7-kb c-jun RNA transcript. As the melanocytes were cultivated in more complete medium, there was a decrease in the expression of the 3.4-kb c-jun RNA transcripts; the transcript was faint when melanocytes were cultivated in complete medium. In the melanomas cultivated in medium with or without

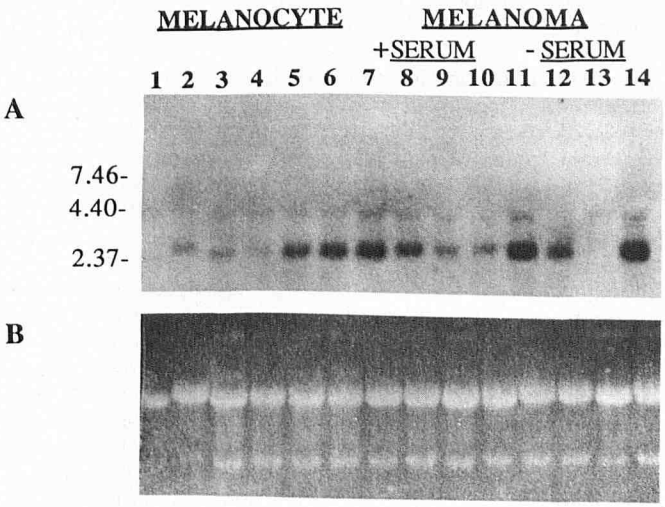

Figure 5. Expression of c-fos RNA transcripts in various growth medium from primary melanocytes and metastatic melanomas. Cells were cultivated and treated for 48 or 72 h in the various conditions described in *Material and Methods*. Ten micrograms of total RNA was hybridized with a ^{32}P-labeled restriction fragment with either c-fos *(A)* or ethidium bromide staining of gel *(B)*. Lane identification, growth conditions, and RNA standards are as described for Fig 3.

Table I. Laser Densimetric Quantification of c-jun, c-fos, and jun-B RNA Transcripts in Primary Melanocytes and Metastatic Melanomas[a]

	c-Fos	c-Jun		jun-B
	2.2 kb	2.7 kb	3.4 kb	2.1 kb
Melanocytes				
P-2	0.23	0.26	ND	NT
P-2	1.00	1.00	1.00	NT
PC	0.61	1.26	0.77	1.00
PCI	0.74	1.08	0.53	1.02
CTS	2.10	0.72	0.16	1.75
C	2.81	0.74	0.12	2.20
Melanomas				
c81-46a −	6.68	0.35	ND	2.33
+	3.81	0.40	ND	0.55
c81-46c −	4.16	0.41	ND	2.45
+	2.81	0.28	ND	1.74
c81-61 −	0.52	0.23	0.02	1.36
+	1.77	1.17	0.98	2.60
c83-2cy −	11.00	0.99	0.40	5.26
+	1.87	0.31	ND	4.64

[a] Primary melanocytes were cultivated in various growth conditions. Metastatic melanoma cell strains were cultivated in medium without or with serum. Values are standardized to c-jun and c-fos RNA transcript expression levels in primary melanocytes cultivated in plain medium for 48 h. Values for jun-B are standardized to jun-B RNA transcript expression levels in primary melanocytes cultivated in plain medium with calcium ions for 48 h. ND, not detected; NT, not tested.

serum, the 3.4-kb c-jun RNA transcript was only detected in the cell strains c83-2cy and c81-61. A 3.4-kb c-jun RNA transcript was detected in c83-2cy cell strain cultivated in medium without serum, but the 3.4-kb c-jun RNA transcript was undetectable when the cells were cultivated in medium with serum. The 3.4-kb c-jun RNA transcript was induced (49.0 times) in c81-61 cells cultivated in medium with serum compared to medium without serum.

Northern analysis of primary melanocytes cultivated in various growth medium for 48 h detected a 2.1-kb jun-B RNA transcript (Fig 4). In quiescent neonatal melanocytes (melanocytes cultivated in plain medium supplemented with Ca^{++} or with Ca^{++} and insulin), a similar level of jun-B RNA transcripts were detected. An increase in jun-B RNA transcript expression levels was detected in proliferating melanocytes cultivated in either complete medium without serum and TPA (0.75 times) or complete medium (1.20 times).

Expression of jun-B RNA transcript levels differed in metastatic melanomas. Melanoma cell strain c81-61 expressed higher levels of jun-B RNA transcripts (0.91 times) when cultivated in medium with serum compared to medium without serum. Repression of jun-B RNA transcript expression levels was observed in the other three melanomas [c81-46a (0.77 times), c81-46c (0.29 times), and c83-2cy (0.12 times)] cultivated in medium with serum compared to medium without serum.

A single 2.2-kb c-fos RNA transcript was detected in primary melanocytes cultivated in the various growth media for 48 h (Fig 5). There was no detectable expression of c-fos RNA transcripts in melanocytes cultivated in medium without any growth factors (plain medium) for 3 d. However, there was an increase in c-fos RNA transcript expression as the melanocytes were cultivated in more complete medium for 48 h. There was a similar low-level of c-fos RNA transcripts in melanocytes cultivated in plain medium or supplemented with Ca^{++}, or with Ca^{++} and insulin for 48 h. For melanocytes cultivated in either complete medium without TPA and serum or complete medium, there was an increase (2.1 times and 2.81 times, respectively) in c-fos RNA transcript expression compared to melanocytes cultivated in plain medium.

The level of expression of c-fos RNA transcripts was different for each melanoma cell strain. Cell strain c81-61 RNA transcript expression level was induced (3.40 times) when cultivated in medium with serum compared to medium without serum. In contrast, the c-fos RNA transcript expression level was decreased in the other three melanomas [c81-46a (0.43 times), c81-46c (0.31 times), and c83-2cy (0.83 times)] when the cells were cultivated in medium with serum compared to medium without serum.

DISCUSSION

We have investigated the expression of c-jun, jun-B, and c-fos RNA transcripts in human primary melanocytes and metastatic melanomas. The initial characterization of the effect of various growth conditions on melanocyte proliferation and the expression of these oncogenes has shown that as human melanocytes are cultivated in progressively more complete medium (i.e., more proliferative state of growth), expression of jun-B and c-fos RNA transcripts was induced. The highest jun-B and c-fos RNA transcript expression levels occurred in medium in which melanocyte number increased.

The regulation of c-jun RNA transcript expression levels in melanocytes was different than the jun-B and c-fos RNA transcript expression levels. The 2.7-kb c-jun RNA transcript was constitutively expressed in melanocytes. Only when the melanocytes were cultivated in medium that resulted in cell death was the 2.7-kb c-jun RNA transcript level reduced. Otherwise, the 2.7-kb c-jun RNA transcript was expressed at a high level. However, the expression of the 3.4-kb c-jun RNA transcript was inhibited as the melanocytes were cultivated in a more complete medium. This second 3.4-kb c-jun RNA transcript has been shown to be due to the utilization of another polyadenylation site [36]. The expression of c-jun RNA transcript in melanocytes appears to be under a relaxed control and easily inducible. These data are consistent with the theory that normal human melanocytes reside in a quiescent state with a high expression level of c-jun RNA transcript.

The expression of c-jun, jun-B, and c-fos proto-oncogenes in metastatic melanomas was different and demonstrated three patterns. In the first case (c81-46a), c-jun RNA transcripts were expressed at a similar level when the melanoma was cultivated in medium with or without serum. This type of tumor may represent one stage of metastatic melanoma. In the second case (c83-2cy and c81-46c), c-jun, jun-B, and c-fos RNA transcripts were repressed and may be reflective of another stage of metastatic melanoma. In the third case (c81-61), c-jun, jun-B, and c-fos RNA transcripts were inducible. This tumor was initially isolated from a pregnant woman and may have been influenced by exogenous growth factors. Evidence of hormonal influence on melanoma proliferation has been published [28,29]. We speculate that this tumor may exist only in rich growth conditions in vitro (i.e., tissue culture with 10% serum) or in vivo (i.e., pregnancy). Support for this speculation comes from the observation that cultivation of this tumor in medium without serum for a short time (> 48 h) led to cell quiescence and death (unpublished data).

The expression of the c-jun and c-fos genes in most other human tumors has been shown to be similar. Both c-fos and c-jun RNA transcript levels are low in normal or benign cells and are high in transformed or malignant cells [30–32]. In contrast, the expression of c-jun, jun-B, and c-fos RNA transcripts in primary neonatal melanocytes was unique. The expression of c-jun RNA transcripts was elevated and both jun-B and c-fos RNA transcripts were reduced in melanocytes compared to metastatic melanomas. High expression levels of c-jun RNA transcripts and low expression levels of jun-B and c-fos RNA transcripts were detected in melanocytes cultivated in non-proliferating conditions.

Elevated c-jun RNA transcripts have been investigated in cells induced to differentiated by the expression of c-Ha-ras oncogene. Human and murine cell lines have been observed to differentiate following transfection or retroviral infection of these cell lines with an activated c-Ha-ras oncogene. Following c-Ha-ras retroviral infection of a human medullary thyroid carcinoma cell line (TT), higher c-jun RNA transcripts and AP-1 protein DNA binding activities were observed [37]. Using a gel mobility shift assay, no differences in AP2, AP3, Sp1, or NF1/CTF DNA binding activity were observed in the nuclear extracts from c-Ha-ras–infected TT cells. Similar studies were carried out on F9 embryonal carcinoma cells

[38]. The expression of an activated c-Ha-ras oncogene in F9 cells induced cell differentiation. F9 cell differentiation markers (stage-specific embryonic antigen, cytoplasmic laminin, and plasminogen activator) protein expression were observed to correlate with the c-Ha-ras or c-jun oncogene-induced differentiation. We speculate that the overexpression of the c-jun proto-oncogene may play a role in inducing cell quiescence or cell differentiation in human primary melanocytes. Further investigation into the overexpression of c-jun oncogene is required to elucidate the effect of the c-jun oncogene in human melanocytes.

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