Differential Expression of Protooncogenes Related to Transformation and Cancer Progression in Rat Myoblasts

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ABSTRACT

We previously derived, from a nonmalignant clonal line of rat myogenic cells (L6al), two sublines which have lost the capacity to differentiate, the M4 cell of low malignancy and the RMS4 cell of high malignancy. In the present study it is shown that 14 of 15 protooncogenes analyzed exhibit detectable levels of transcripts during L6al cell proliferation. When L6al cells form myotubes, the levels of c-abl, c-myb, and c-Ha-ras transcripts remain unchanged, the level of c-N-ras RNA is augmented, the level of c-erbB RNA is markedly reduced, and all other c-onc transcripts (c-erbA, c-sis, c-src, c-fes, c-fms, c-fos, c-myc, c-Ki-ras, and the putative tyrosine kinase transcript of the c-fgr gene) become hardy, if at all, detectable. Surprisingly, when the three cell types are growing at similar rates only, one protooncogene (c-mos) is not detected at detectable levels in L6al, two others (c-fos, c-erbA) are not expressed in M4 or in RMS4, and three additional ones (c-erbB, c-sis, c-src) are expressed in M4 but not in RMS4. Moreover the level of c-fes RNAs is markedly lower in RMS4 than in M4 or L6al. By contrast, the level of two c-Ki-ras 5.4- and 2.2-kilobase transcripts is lower in M4 and L6al than in RMS4, and the latter contains another abundant c-Ki-ras 3.8-kilobase transcript which is hardly detectable in M4 and not at all in L6al. These data suggest an activation of the c-Ki-ras gene in the malignant myoblasts and some relationship between the progression of malignancy and inactivation of certain other c-onc genes.

INTRODUCTION

A growing body of evidence strongly suggests that neoplastic transformation can be caused by abnormal activation of protooncogenes (c-onc genes), as a result of chromosomal rearrangement, c-onc gene amplification, point mutation, or some other type of alteration (see Refs. 1–5 for review). It is generally admitted that a better knowledge of the physiological functions of protooncogenes products will greatly contribute to the elucidation of the mechanisms of carcinogenesis, especially in view of recent data showing that several c-onc genes (and possibly most of them) are implicated in the control of normal cell growth (see Ref. 6 for review). The proteins encoded by the c-sis, c-erbB, and c-fms genes are respectively related to the platelet-derived growth factor (7, 8), the receptor for the epithelial growth factor (9), and the receptor for the mononuclear phagocyte growth factor, CSF-1 (10). The expression of the c-myc (11–13) and c-Ki-ras (12, 14) genes is enhanced when quiescent cells in culture are induced to proliferate (14) or during liver regeneration (15). A rapid but transient expression of the c-fos gene precedes that of c-myc when fibroblasts or lymphocytes are stimulated by growth inducers (16–18) and both c-myc and c-sis genes are coordinately expressed in developing human placenta (19).

However, the relationship between these effects and cell proliferation is far from being elucidated. Recent data show that the levels of c-myc RNA and c-myc protein synthesis are independent of the cell cycle in human or avian cells in culture (20, 21). Furthermore, other recent findings (which are cited in the "Discussion") indicate that changes in the expression of protooncogenes like c-myc, c-fos, and c-fms can be related to the process of differentiation rather than to a growth-dependent phenomenon, or vice versa, depending on the type of cell and some of our data support this view. Therefore in order to determine the possible role of c-onc genes in the genesis and(or) progression of a given type of neoplasm it is interesting to analyze a large spectrum of these genes in model systems consisting of normal cells capable of undergoing a well defined differentiation process compared to transformed derivatives of differing malignant potentials. The c-myc, c-fos, and c-erbB genes were selected for this study because of recent data showing that several c-onc genes (and possibly most of them) are implicated in the control of normal cell growth (see Ref. 6 for review). The proteins encoded by the c-sis, c-erbB, and c-fms genes are respectively related to the platelet-derived growth factor (7, 8), the receptor for the epithelial growth factor (9), and the receptor for the mononuclear phagocyte growth factor, CSF-1 (10). The expression of the c-myc (11–13) and c-Ki-ras (12, 14) genes is enhanced when quiescent cells in culture are induced to proliferate (14) or during liver regeneration (15). A rapid but transient expression of the c-fos gene precedes that of c-myc when fibroblasts or lymphocytes are stimulated by growth inducers (16–18) and both c-myc and c-sis genes are coordinately expressed in developing human placenta (19).

MATERIALS AND METHODS

Cell Culture. The L6al cell line, obtained in our laboratory by subcloning the L6 cell line originally isolated by Yaffe (22) was maintained and induced to differentiate under previously described conditions (23, 25). The transformed M4 and RMS4 sublines were passaged and assayed for growth properties and malignant characteristics as indicated previously (24, 27).

Isolation and Blotting Analysis of RNA. Total cell RNA was isolated by the thiocyanate guanidine procedure (28) and selected for polyadenylate containing sequences by retention on oligodeoxythymidylate cellulose under previously specified conditions (25); gel electrophoresis and Northern blot analysis with nick-translated 32P-labeled recombinant DNA probes were essentially as described previously (29). Blots were prehybridized for 2–3 h at 42°C in 20–50 ml of 5× SSC.3× 50 mM sodium phosphate, pH 7.0, containing 50% deionized formamide, 0.2% SDS, 0.5 mM EDTA, 5× Denhardt’s solution and denatured, sonicated salmon sperm DNA (100 µg/ml). Hybridizations were performed for 24–40 h under the same conditions with 0.1–0.2 µg of highly labeled probes (2–3 × 106 cpm/µg). Blots were washed for 3 × 15 min with 250 ml of 2X SSC-0.5% SDS with gentle shaking at room temperature and then given a final wash of 30 min in 0.1× SSC-0.1% SDS at 65°C for the murine c-onc probes and at 55°C for then nonmurine probes.

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The abbreviations used are: 1 × SSC, 0.15 M NaCl-0.015 M sodium citrate; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.
Quantitation of c-onc transcripts was determined by densitometric scanning (with a Vernon photometric recorder) of autoradiographs from dot blots and the areas under each peak were integrated.

Isolation and Blotting Analysis of Genomic DNA. For isolation of cellular DNA, cells were lysed in the same thiocyanate buffer as for RNA extraction. The lysate was layered over a cushion of 5.7 M CsCl-0.1 M EDTA, pH 7.0, in a nitrate cellulose tube convenient for the cellular DNA, cells were lysed in the same thiocyanate buffer as for scanning (with a Vernon photometric recorder) of autoradiographs. Quantitation of c-onc transcripts was determined by densitometric analysis of the autoradiographs.

Isolation and Blotting Analysis of Genomic DNA. For isolation of genomic DNA, cells were lysed in the same thiocyanate buffer as for scanning (with a Vernon photometric recorder) of autoradiographs. Quantitation of c-onc transcripts was determined by densitometric analysis of the autoradiographs.

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Characteristics of the 3 Cell Lines

Our previous studies showed that the L6a1 cells exhibit no malignant characteristics and retain the capacity to differentiate whereas the M4 and RMS4 transformants have lost this capacity and differ from each other in terms of cell morphology, karyotypic alterations, growth potential in semisolid medium, and tumorigenic potential in vivo (24, 27). These differential characteristics remain unchanged until the present time as demonstrated by recent assays. As shown in Fig. 1A, when plated in 10% FCS the 3 cell lines exhibit similar growth rates for at least 50-60 h and the same is true for DNA synthesis (data not shown). However, when reaching confluence the L6a1 cells are growth arrested while the M4 and RMS4 cells are not. Furthermore, when plated in a low serum medium the transformed cells proliferate almost at the same rates as in 10% FCS whereas the L6a1 cells stay stationary or nearly so (see Fig. 1B).

Other recent assays confirmed the different malignant potential of the M4 and RMS4 cells. In vitro, 48–50% of the RMS4 cells assayed were found capable of forming colonies in soft agar compared to 10–11% for M4 cells and at most 0.01% for L6a1 cells. In vivo, no tumor was observed in 10 baby rats inoculated s.c. with 5 x 10^6 L6a1 cells whereas tumors that developed as typical rhabdomyosarcomas became palpable after 25–30 days in 4 of 5 animals inoculated with 5 x 10^6 M4 cells and after 4–6 days in 10 of 10 animals inoculated with 2 x 10^6 RMS4 cells.

Quantitative Evaluations of c-onc-related Transcripts

In order to obtain a reliable comparison of the various c-onc transcripts, in the L6a1, M4, and RMS4 cells, all were plated in 10% FCS and collected 40–42 h later when the 3 cell lines still exhibit similar growth rates. Exponentially growing L6a1 myoblasts were also compared with terminally differentiating myotubes collected 4–5 days after the transfer in 2% FCS. Polyadenylate-containing RNAs were purified and the same RNA batches were analyzed by the dot blot techniques with the various c-onc-related probes, using as internal control the probe which recognizes the cytoskeletal actin as well as the muscle-specific α-actin transcripts and the dot blot autoradiograms were quantitatively evaluated by scanning with a Vernon densitometer. To simplify presentation of the data we developed an arbitrary scale of increasing hybridization signal intensities illustrated in Fig. 2. The data recapitulated in Table 1 may be considered as correct because they were confirmed in 2 or 3 separate experiments. Moreover, except for a few discrepancies, they are consistent with the results of Northern blot analyses.

Analysis of c-onc-related Transcripts

Northern blot analysis of c-abl-, c-myc-, and c-Ha-ras-related RNAs (not shown) showed the same patterns as previously described (47–49) and confirmed that similar levels of all these transcripts are found in M4 and RMS4 cells as well as in L6a1 cells, either myoblasts or myotubes. The patterns of all other c-onc-related transcripts compared to actin-specific transcripts are shown in Fig. 3. They may be summarized as follows.

Actin-related RNAs. One 2.2-kilobase transcript, which is similarly abundant in M4, RMS4, and L6a1 myoblasts, is reduced to very low levels in myotubes. Another 1.65-kilobase transcript is found only in myotubes. They correspond to β- and γ-actin RNAs and α-actin RNA, respectively (32, 33).

c-myc-related RNA. A 2.6-kilobase transcript exhibits equivalent high levels in M4, RMS4, and L6a1 myoblasts and very low levels in myotubes.

c-src-related RNA. Two transcripts (6.8 and 4.2 kilobases) exhibit similar levels in M4 and L6a1 myoblasts but are unde-
expression of c-onc in malignant progression

Fig. 2. A, scale of intensities of dot blot hybridization signals. ±— to ++++ correspond to the indicated numbers (in arbitrary units) obtained by densitometric scanning of autoradiograms, using a Vernon photometric recorder. B, dot blot hybridization of the Ki-ras and actin probes to cellular RNAs. The indicated amounts of polyadenylate-containing RNA were spotted onto nitrocellulose paper and hybridized with the 32P-labeled probe (about 10⁶ cpm/ml) for 30 h (see "Materials and Methods" and references cited). Exposure times of autoradiograms were typically 2 days for the Ki-ras probe and 5 h for the actin probe. Sources of RNA were: Lane 1, proliferating L6nl cells (2 days in 10% PCS); Lane 2, differentiating L6nl cells (5 days in 2% PCS, >90% fused cells); Lane 3, M4 cells (2 days in 10% PCS); Lane 4, RMS4 cells (2 days in 10% PCS).

Table 1 Quantitative evaluations of c-onc-related transcripts in L6nl myoblasts, L6nl myotubes, and M4 and RMS4 cells

L6nl myoblasts and M4 and RMS4 cells were collected after 48 h of culture in 10% FCS. L6nl myoblasts were collected at the final stage of differentiation (after 4–5 days in 2% PCS) when more than 90% of the cells are fused. Polyadenylate-containing RNA was purified and aliquot samples were subjected to dot blot hybridizations with each of the 15 c-onc probes or with actin-specific probe, as indicated in the legend to Fig. 2. ±— to ++++, relative intensities of hybridization signals evaluated by densitometer scanning. 0, no signal or signal close to the background noise.

Normal cells Tumorigenic cells

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>L6nl myoblast</th>
<th>L6nl myotube</th>
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<td>fgr</td>
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tectable in RMS4 and in myotubes. The same is found for one c-sis-related 6.3-kilobase transcript. The latter might represent a precursor species or a large mRNA peculiar to the L6 line.

c-fos-related RNA. One 2.2-kilobase transcript very abundant in L6nl myoblasts is hardly or not detectable in M4 and RMS4 cells and in myotubes. The same is found for one c-erba1-related 2.9-kilobase transcript of low abundance.

c-erbb-related RNA. One main 5.5-kilobase transcript is abundant in L6nl myoblasts, less abundant in M4 cell and myotubes, and undetectable in RMS4 cells; one minor 8.2-kilobase transcript (presumably a precursor) is found in both M4 and L6nl myoblasts but not in myotubes and RMS4 cells.

c-N-ras-related RNA. One 3.8-kilobase transcript, the same as described previously (50), exhibits similar high levels in M4, RMS4, and L6nl myotubes and reproducibly lower levels in L6nl myoblasts.

c-Ki-ras-related RNA. Two transcripts (5.4 and 2.2 kilobases), similar to those described elsewhere (47), are markedly more abundant in RMS4 than in M4 and L6nl myoblasts and exhibit much lower levels in myotubes. Another 3.8-kilobase transcript is very abundant in RMS4 cells, hardly detectable in M4 cells, and undetectable in L6nl cells.

c-fms-related RNA. One main 3.7-kilobase transcript, the same as in mouse placenta (51), and 1 minor 2.0-kilobase transcript is equally abundant in M4, RMS4, and L6nl myoblasts and not detectable in myotubes.

c-fes-related RNA. Two transcripts (3.2 and 1.8 kilobases), probably the same as described previously (41), exhibit equivalent levels in M4 and L6nl myoblasts and constantly lower levels in RMS4 cells but are not found in myotubes.

c-fgr-related RNA. The transcript pattern is consistent with the dual origin of v-fgr which contains one portion of actin gene and one tyrosine kinase-related sequence (52). One 2.2-kilobase transcript, which is equally abundant in all the proliferating myoblasts and practically missing in myotubes corresponds to cytoskeletal actin RNAs; one 1.65-kilobase transcript found only in myotubes corresponds to α-actin RNA. The 3.6-kilobase transcript which exhibits equivalent levels in all the myoblasts and is not found in myotubes may represent the tyrosine kinase-specific RNA.

c-Ki-ras-related Transcripts Accumulate in the Malignant Cells Kept in Low Serum

It was of interest to determine whether the use of low serum which allows the differentiation of L6nl cells, accompanied by a drastic reduction in the level of c-Ki-ras, c-myc, or other c-onc transcripts, may cause comparable effects in the malignant cells. Some typical results of a time course analysis of c-Ki-ras and c-myc transcripts compared to actin-specific transcripts are shown in Fig. 4. It may be seen that in 2% FCS, the level of c-myc RNA found in RMS4 cells, remains essentially stable in the course of time. The level of c-Ki-ras-related RNAs increases significantly mainly because the additional 3.8-kilobase transcript tends to accumulate. By contrast the level of cytoskeletal actin RNA decreases progressively and, as expected, no muscle-specific actin RNA is made.

Southern Blot Analysis Reverses No Obvious c-onc Gene Alteration

In order to determine whether the dramatic changes in the expression of various protooncogenes, which appear to result from the neoplastic transformation of L6nl cell, could be related to obvious genomic alterations, 9 of these genes were analyzed by the technique of Southern. It is shown in Fig. 5
that all the DNA sequences related to the c-fos, c-myc, c-N-ras, and c-Ki-ras genes are associated with genomic fragments of identical mobilities and intensities of the hybridization signals do not markedly differ in the DNA from M4 and RMS4 cells compared to L6a1 cells or normal spleen cells. The same was found true for the c-fes, c-Ha-ras, c-erbA, c-erbB, and c-fms genes (data not shown). This renders unlikely the occurrence of large sequence rearrangements or gross amplification involv-
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Fig. 5. Southern blot analysis of c-Na-roc, c-myc, c-Ki-ras, and c-fos genomic sequences. Samples of 10 μg of high molecular weight DNA were digested with the restriction endonucleases, subjected to electrophoresis in 0.8% agarose gels, transferred to nitrocellulose, and hybridized to the 32P-labeled probes in the conditions utilized for analysis of c-onc transcripts. Exposure time of autoradiograms was 36 h. Sources of DNA: Lane 1, L6al myoblasts; Lane 2, L6al myotubes (4 days in 2% FCS); Lane 3, M4 cells; Lane 4, RMS4 cells; Lane 5, normal rat spleen. λ-HindIII digest was run in parallel and the sizes of the reference fragments are indicated in kilobase pairs.

DISCUSSION

To our knowledge this study is the first to investigate the expression of many protooncogenes in tissue-cultured cells capable of undergoing differentiation, compared to transformants of differing malignant potentials. Since our analysis was limited to RNA species accumulating at detectable levels it did not address the question of the mechanism, either transcriptional or posttranscriptional (or both), which regulates this expression. In fact, although important in itself, this question is not of great consequence in the present case because it is reasonable to assume that dramatic changes at the level of c-onc transcripts will probably cause the same effect whatever the mechanism. More crucial questions about the corresponding c-onc proteins cannot be answered until analyses of these proteins are completed but the necessary reactivies are not all available yet. For example, as shown above, when L6al cells form myotubes the level of c-Ha-ras RNA is not modified while that of c-N-ras RNA is augmented and that of c-Ki-ras RNA is drastically reduced. It would be of evident interest to determine whether or not these changes are reflected at the level of c-Ki-ras and c-N-ras proteins. Unfortunately, at the moment, the only antibody which we can use for that purpose (obtained from Dr. Papageorge, National Cancer Institute, Bethesda, MD) cannot discriminate the N-ras from the Ki-ras protein.

One main objective of the present work was to search for activation of various protooncogenes in the neoplastic transformants. In fact, as shown above, only the c-Ki-ras gene (and possibly also the c-N-ras) appears to be abnormally expressed in the transformants mainly because of an additional Ki-ras-related transcript which is very abundant in RMS4 cells, hardly detectable in M4 cells, and seemingly absent from L6al cells. This suggested an oncogenic activation of the Ki-ras gene and transfection experiments were undertaken to test this possibility. Colonies of transformed cells were obtained in mouse 3T3 cell cultures treated with RMS4 cell DNA and preliminary results indicate that these transformed cells harbor additional DNA sequences related to the Ki-ras gene, but further work is needed to characterize these sequences. An oncogenic activation of the c-Ki-ras gene does not exclude a cooperative action of other protooncogenes, in particular c-N-ras, even if these protooncogenes are not obviously over expressed in the neoplastic transformants. The same may be true for other cellular genes, hitherto not identified. In the last years evidence for multigenic activation resulting from experimental carcinogenesis was repeatedly reported (see Refs. 56–58 for review) and it was found in our laboratory that many distinct transcription units appear to be tumor activated in a variety of human neoplasias (57, 58). Moreover, we previously showed that the RMS4 cells contain several hundred putative mRNA species that are missing or very rare in proliferating L6al cells whereas the latter contain about 2000 RNA species that are missing or very rare in RMS4 cells (27). This suggested that in those tumor cells a whole multigenic set is activated and a larger multigenic set is inactivated.

In view of current concepts it could be expected that the tumor-activated set should more probably include various protooncogenes than the tumor-inactivated one. It was therefore surprising to discover, repeatedly, that in fact the opposite is true. As shown in Table I and Fig. 2, of the 15 protooncogenes investigated, 6 (c-mos, c-fos, c-erbA, c-erbB, c-src, c-sis) appear to be totally inactive in the RMS4 transformants (at least they
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exhibit no detectable RNA amount whereas only one (c-mos) appears to be totally inactive in proliferating L6α1 cells. The difference is particularly striking (and of obvious significance) for the c-fos and c-erbB genes since in the parental stem cells c-fos and c-erbB transcripts are much more abundant than c-erbA, c-src, and c-sis transcripts. Strikingly also, of the 5 protooncogenes that are expressed in L6α1 stem cells and appear to be tumor inactivated in the malignant RMS4 transformants, only 2 (c-fos and c-erbB) appear to be similarly inactivated in the low malignant M4 transformants. This tentatively suggests a possible relationship between the progress of neoplastic potential and a stepwise inactivation of certain protooncogenes.

It should be underlined that in all those comparative assays the various cell samples were collected during the phase of exponential growth when the 3 cell lines exhibit similar multiplication rates. Therefore, the conspicuous changes at the level of various c-onc genes transcripts observed in the neoplastic myoblasts cannot likely be due to a growth-dependent phenomenon. Were it the case, the increased proliferation capacity of the tumor cells may well explain an activation of protooncogenes as well as an activation of one or two ras gene(s). Nonetheless evident that its generalization to other types of neoplasias could provide a new approach to the problem of cancer progression.

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