Quantitative Polymerase Chain Reaction of Lysyl Oxidase mRNA in Malignantly Transformed Human Cell Lines Demonstrates That Their Low Lysyl Oxidase Activity Is Due to Low Quantities of Its mRNA and Low Levels of Transcription of the Respective Gene*

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Lysyl oxidase (EC 1.4.3.13), an extracellular copper amino oxidase, initiates the cross-linking of collagens and elastin by catalyzing oxidative deamination of the ε-amino group in certain lysine and hydroxylysine residues. We developed here a polymerase chain reaction (PCR) method for the quantification of lysyl oxidase mRNA in which a synthetic RNA is used as an internal standard for coamplification with the targeted mRNA. The amount of lysyl oxidase mRNA when studied by Northern blot analysis and the number of lysyl oxidase mRNA molecules when determined by the quantitative PCR method were found to be markedly low in various malignantly transformed cell lines relative to control cell lines, quantitative PCR indicating values of about 2–10% of those in the controls. No difference was found in the number of β-actin mRNA molecules between the transformed cells and the controls. Nuclear runoff experiments indicated that most if not all of the decrease in the number of lysyl oxidase mRNA molecules can be explained by diminished transcription of the respective gene.

Lysyl oxidase (EC 1.4.3.13), an extracellular copper enzyme, initiates the cross-linking of collagens and elastin by catalyzing oxidative deamination of the ε-amino group in certain lysine and hydroxylysine residues of collagens and lysine residues of elastin (for reviews, see Refs. 1 and 2). Molecular cloning and complete cDNA-derived amino acid sequences have been reported for the rat (3, 4), human (5, 6), and chick (7) enzymes, which were found to be synthesized in precursor forms of 411, 417, and 420 amino acids, respectively. The human lysyl oxidase gene is located on chromosome 5 (8–10), both genes consisting of seven exons (11, 12). Increased lysyl oxidase activity has been reported in fibrotic disorders (1), while a deficiency is found in two X-linked, recessively inherited human disorders, the occipital horn syndrome and Menkes syndrome, and in the X-linked, recessively inherited Mckusick-Kaufman syndrome (13, 14). In these X-linked disorders, the low enzyme activity appears to be secondary to abnormalities in copper metabolism. Lysyl oxidase activity is markedly low in the culture medium of many malignantly transformed human cell lines (15). The cDNA-derived amino acid sequence of the mouse ras recision gene, rrg (17), has been found to match that of rat lysyl oxidase (18), suggesting that rrg and lysyl oxidase are identical. The levels of rrg mRNA (17) and lysyl oxidase activity (18) are markedly decreased in NIH 3T3 cells transformed by LTR-c-Ha-ras compared with those in nontransformed NIH 3T3 or in cells after reversion following prolonged treatment with interferon-β (17). Transfection of the revertants with antisense rrg constructs leads to a transformed morphology again, and the cells become tumorigenic in nude mice (17).

The purpose of this work was to explore further the reasons for the low lysyl oxidase activity observed in the culture medium of malignantly transformed cells. For this purpose, we developed a PCR method for the quantification of lysyl oxidase mRNA in which a synthetic RNA (cRNA) is used as an internal standard for coamplification with the target mRNA. We also studied the amount of lysyl oxidase mRNA by Northern blotting and the production of this mRNA in vitro nuclear runoff experiments.

MATERIALS AND METHODS

Cell Culture—The cultured human cell lines were embryonal skin (HES) and lung (HEL, WI-38) fibroblasts and adult skin (2090, 9505, 9011) fibroblasts, SV40 virus-transformed WI-38 cells (VA-13), fibrosarcoma cells (HT-1080), embryonal rhabdomyosarcoma cells (RD), choriocarcinoma cells (JEG-3), and melanoma cells (G-361). The cells were cultured at 37 °C at 5% CO2 in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum, 100 units/ml penicillin and streptomycin.

RNA Preparations and Northern Hybridization—Total cytoplasmic RNA was isolated from cultured cells by acid guanidinium thiocyanatephenol-chloroform extraction (19) and stored dry at −70 °C until used. The RNA yield was measured by absorbance at 260 nm. Electrophoresis of total RNA was performed on a 1.0% agarose gel containing 2M formaldehyde, and RNA was transferred to a nitrocellulose filter and hybridized with 32P-labeled HLO20 cDNA for human lysyl oxidase (5) and glyceraldehyde 3-phosphate dehydrogenase (20).

Assay for Lysyl Oxidase Activity—Lysyl oxidase activity was assayed from the conditioned medium. A tritiated, insoluble elastin substrate was prepared from the aortae of 16-day-old chick embryos, which had been pulsed in organ culture with L-4,5-(3H)lysine in the presence of β-aminopropionitrile (21).

Internal and External Controls—The lysyl oxidase and β-actin con

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1 The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; tip, base pair(s).
Lysyl Oxidase mRNA in Malignantly Transformed Cells

**TABLE I**

| mRNA species     | Size of PCR product | 2000 |
|------------------|---------------------|------|
|                  | bp                  |      |
| Lysyl oxidase    |                     |      |
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**RESULTS**

**Northern Blot Analysis**—The amount of lysyl oxidase mRNA in five malignantly transformed human cell lines and six controls was determined by Northern blotting. HLO20, a cDNA probe for lysyl oxidase (5), hybridizes to multiple mRNAs, the sizes being 5.5, 4.3, 2.4, and 2.0 kilobases. The 4.3-kilobase band, corresponding to the major species of the human lysyl oxidase mRNA is shown. cRNA molecules. Serial dilutions of the appropriate ratios of cRNA to cDNA were subsequently prepared and amplified. 10 μl of each PCR product was separated on 6% PAGE, dried, and autoradiographed. The bands corresponding to each specific PCR product were then excised from the dried gels, and the amount of radioactivity incorporated was determined by liquid scintillation counting. The cRNA incorporation data for each experiment were plotted, and the linear portion of the curve was identified. Linear regression with a y intercept of zero was performed on the data that were linear. The slope of the regression line was used to compute the numbers of endogenous molecules per program of total cellular RNA. A few experiments were deemed inconclusive since the correlation of the control data with the regression line was poor (R² < 0.75). For the dilutions over which the control data were linear, the computed number of endogenous molecules usually also demonstrated linearity.

**Quantitative Analysis**—Preliminary PCRs were performed to establish the number of control molecules necessary to obtain signals of approximately equal intensities from the endogenous cDNA and control
oxidase probe must have been due to a marked reduction in the amount of the lysyl oxidase mRNA.

Quantitative PCR for Lysyl Oxidase mRNA with Internal Standard—To quantify the lysyl oxidase mRNA in the transformed cell lines, a PCR-based assay was developed. An internal standard containing a deletion of 60 bp in the middle of the lysyl oxidase cDNA was constructed. In vitro transcribed RNA (cRNA) was generated from this control construct. Known amounts of the cRNA were mixed together with known amounts of total RNA isolated from cultured cells, and cDNA synthesis, PCR, and PAGE analysis were carried out. The PCR products obtained with the cDNAs derived from the cRNA and the cellular RNA differed in size by 60 bp and were therefore easy to distinguish on PAGE. Fig. 2 shows an example of the PCR products specific to lysyl oxidase mRNA and the cRNA. In this experiment, the cDNAs were synthesized under standard conditions with 0.93 μg of total cellular RNA from the control cell line 9011 and 10.96 μg of total cellular RNA from the fibrosarcoma cell line HT-1080. In both cases, the RNA samples also contained 8.44 × 10^7 molecules of the lysyl oxidase cRNA as an internal standard. Serial 1:2 dilutions were made, and PCR amplifications were carried out. The internal standard had two functions. First, it served as an internal control for the reverse transcription and PCR amplification reactions, and second, it was used to generate a standard curve for quantifying the target mRNA in experimental samples. Since the same primers were used in the PCR for both templates, there were no primer efficiency differences between the standard and the target RNAs. Different dilutions of cDNA synthesis mixtures containing both the target mRNA and standard cRNA were coamplified in the same tube in the exponential phase, and the amount of target mRNA was determined by interpolating against the cRNA standard curve. The amplification efficiencies were determined as described by Wang et al. (27). The efficiency was 40–46% (n = 6) for cDNA derived from RNA isolated from three cell lines and 41–47% (n = 6) for cDNA derived from the internal standard. The corresponding values for β-actin mRNA were 32–40% and 35–41%.

Quantities of Lysyl Oxidase mRNA in Malignantly Transformed and Control Cell Lines—As previously reported (16), the lysyl oxidase activity of all the transformed cell lines was very low, being below the limit of accurate measurement (Table II). The number of lysyl oxidase mRNA molecules per picogram of total cellular RNA varied between 340 and 440 in four control cell lines, but the numbers in the transformed cell lines were only about 2–10% of these (p < 0.0005), being 7–40 molecules/pg (Table II). No differences in the number of β-actin mRNA molecules were found between the transformed cells and the controls (Table II). The number of mRNA molecules for the Rho1 chain of type III procollagen had decreased, although to a smaller extent than for lysyl oxidase; the values in the four control cell lines ranged from 380 to 980 molecules/pg RNA, whereas that value in the VA-13 cells was 72 molecules and that in the RD cells was 126 molecules/pg RNA (details not shown).

Nuclear Runoff Transcription—The levels of transcription of the lysyl oxidase gene were measured in different cell lines using nuclear runoff assays. Quantification of pre-mRNA molecules in such experiments demonstrated that the level of transcription of the lysyl oxidase gene is markedly lower (p < 0.0005) in transformed cells than in the controls (Table II). The decrease in the transcription level was not quite as marked, however, as the decrease in the amounts of lysyl oxidase mRNA molecules and enzyme activity.

**DISCUSSION**

The quantitative PCR method developed here for human lysyl oxidase mRNA follows the principles described by Wang et al. (27). The specific mRNA and an internal standard cRNA containing a 60-bp deletion are coamplified in the same reaction and with the same primers, and the PCR products are separated by PAGE and quantified. The numbers of endogenous mRNA molecules can then be computed from the curve determined for the internal standard.

The data obtained with the quantitative PCR indicated that the number of lysyl oxidase mRNA molecules per picogram of total RNA was about 340–440 in the four control cell lines, the mean being about 8% of the mean for the values of about 2000–6600 molecules/pg determined for the mRNA molecules that encode β-actin and about 60% of the mean for the values of 380–980 molecules/pg for those encoding the proc1 chain of type III procollagen. The last-mentioned value does not indicate a ratio of the number of mRNA molecules for lysyl oxidase

| Cell line                        | Lysyl oxidase activity | Number of lysyl oxidase mRNA molecules | Number of β-actin mRNA molecules |
|----------------------------------|------------------------|----------------------------------------|---------------------------------|
|                                  | dpm/10^6 cells         | molecules/pg RNA                       | molecules/pg RNA                |
| Control cells                    |                        |                                        |                                 |
| Embryonal skin fibroblasts (HES) | 2600                   | 442 ± 25 [4]                           | 5180 ± 1150 [7]                 |
| Adult skin fibroblasts           |                        |                                        |                                 |
| 9011                             | 1730                   | 432 ± 121 [13]                         | 2000 ± 400 [9]                  |
| 9505                             | 1030                   | 363 ± 95 [6]                           | 6610 ± 1690 [7]                 |
| 20009                            | 1340                   | 344 ± 47 [13]                          | 5500 ± 1600 [3]                 |
| Sarcoma cells                    |                        |                                        |                                 |
| SV40-transformed WI-38 cells (VA-13) | 60                  | 7 ± 3 [5]                              | 4060 ± 1720 [7]                 |
| Fibrosarcoma cells (HT-1080)     | 20                     | 40 ± 5 [5]                             | 2530 ± 555 [8]                  |
| Embryonal rhadobomyosarcoma cells (RD) | 20                  | 22 ± 4 [11]                            | 2015 ± 1450 [10]                |
| Other transformed cells          |                        |                                        |                                 |
| Choriocarcinoma cells (JEG-3)    | 50                     | 13 ± 4 [3]                             | 2490 ± 443 [10]                 |

| Number of lysyl oxidase mRNA molecules per picogram of total RNA. |
| Mean ± S.D. |
| Number of values used in calculating the mean. |
to the number for its polypeptide substrate because type III procollagen represents only about 15–20% of the total collagen synthesized by cultured skin fibroblasts (see Ref. 28 and references therein). Thus, the number of lysyl oxidase mRNA molecules may be about one-tenth of the number for its polypeptide substrate.

The number of lysyl oxidase mRNA molecules in malignantly transformed cell lines was very low, both when measured by Northern blotting and when assayed with the quantitative PCR. The latter indicated values of about 2–10% of those in the controls. Lysyl oxidase activity in malignantly transformed cell lines has previously been reported as about 10% that in controls (16). The present assays indicated an even lower percentage, but as the activity levels in the transformed cells were below the limit of accurate measurement both in the previous study (16) and here, the actual levels probably do not differ greatly. The magnitude of the decrease in the number of lysyl oxidase mRNA molecules thus appears to be very similar to that in enzyme activity, indicating that the low enzyme activity is due to a pretranslational mechanism.

Nuclear runoff experiments indicated that most if not all of the decrease in the lysyl oxidase mRNA levels can be explained by diminished transcription of the respective gene. However, as the runoff values for the transformed cells were not quite as low as those for the mRNA molecules by comparison with the control cells, the data do not exclude the possibility that decreased stability of the lysyl oxidase mRNA may have contributed to the low mRNA and enzyme activity levels.

The present data obtained with several human tumor cell lines are in good agreement with those reported for the rrg mRNA, which appears to be identical to the lysyl oxidase mRNA in NIH 3T3 cells transformed by LTR-c-Ha-ras (see Introduction). Thus, the findings reported for the LTR-c-Ha-ras-transformed NIH 3T3 cells appear to be similar in a number of tumor cell types. It is of particular interest that the data obtained with the NIH 3T3 cells suggest that the lysyl oxidase gene may have a tumor suppressor activity (17, 18). However, the mechanisms by which this gene may achieve this activity are currently unknown. It is likewise unknown what cis-acting elements in the lysyl oxidase gene and trans-acting factors in the cells are responsible for the low lysyl oxidase mRNA levels in the tumor cells. The promoter region and downstream sequences in the lysyl oxidase gene contain a number of potential binding sites for various transcription factors (12), but the elements involved in the decrease in the transcription of the gene in transformed cells remain to be identified.

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TABLE III
Quantification of lysyl oxidase nuclear RNA

| Cell line                      | Number of lysyl oxidase nuclear RNA molecules | molecules/ pg RNA* |
|-------------------------------|---------------------------------------------|-------------------|
| Control cells                 |                                             |                   |
| Adult skin fibroblasts        | 9011                                        | 85,810 ± 16,710*  |
| 9505                          | 23,170                                      | 5040*             |
| NO5                           | 68,110                                      | 10,940 (4)        |
| Mean                          | 57,890                                      | 30,800 (16)       |
| Transformed cells             |                                             |                   |
| Embryonal rhabdomyosarcoma    | 6000                                        | 720 (3)           |
| cells (rd)                    |                                             |                   |
| SV40-transformed lung fibroblasts (VA-13) | 4090                                      | 560 (3)           |
| Fibrosarcoma cells (HT-1080)  | 22,070                                      | 3870 (3)          |
| Choriocarcinoma cells (JEG-3) | 8010                                        | 2450 (3)          |
| Mean                          | 10,040                                      | 7660 (12)         |

* Number of molecules per picogram nuclear RNA.
* Mean ± S.D.
* Number of values used in calculating the mean.