Correlation of V-src Gene Amplification with the Tumorigenic Phenotype in a Syrian Hamster Embryo Cell Line

Tona M. Gilmer, Pattie W. Lamb, Mitsuo Oshimura, and J. Carl Barrett

Environmental Carcinogenesis Group, Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

A preneoplastic cell line (10W) isolated after treatment of Syrian hamster embryo cells with asbestos was cotransfected with pSV2-neo DNA and Rous sarcoma virus DNA. Six of these colonies contained v-src DNA; however, none of the six initially expressed -src RNA. Five of the clones failed to grow in soft agar (frequency, <10^{-3}). One clone (61) grew in soft agar, but with a low frequency. Three of the clones (41, 61, and 62) were tumorigenic in nude mice and three were nontumorigenic. Cells cloned from soft agar or established from tumor explants expressed the v-src gene. The gene copy number of v-src, which was three to 10 in the original neo clones, was increased approximately 10-fold in the soft agar-derived cell clones and tumor-derived cell lines. Cytogenetic analyses indicated that cells with amplified v-src contained double minute chromosomes. The results suggest that gene amplification influences the expression of the transfected oncogene and is a mechanism which can overcome the initial suppression of transcription of the v-src oncogene in the 10W cell line.

INTRODUCTION

The product of the oncogene v-src, a phosphoprotein (pp60^c), with tyrosine-specific kinase activity, is responsible for transformation of cells by RSV (1-4). RSV can efficiently transform certain normal and immortal cells apparently in a single step, whereas transformation of some normal, rodent cells by transfection with RSV DNA is not as efficient and multiple steps may be needed (5, 6). Our previous work showed that neoplastic transformation of tertiary passage SHE cells by transfection with a genomic clone of RSV occurs with a low frequency and only after a long latency period (5). We suggested that this was due to an initial suppression of the expression of the v-src gene by cellular factors following transfection of RSV DNA into the cells. We did not observe any morphological transformation of the SHE cell colonies cotransfected with RSV and pSV2-neo DNAs, whereas the v-Ha-ras oncogene did induce morphological changes (7). Since the SHE cell colonies senesced, adequate numbers of cells could not be obtained to study the expression of the v-src gene and its regulation. We suggested that the RSV DNA was not expressed in these normal cells even though the same plasmid was expressed in hamster cells which had been immortalized by diethylstilbestrol. The RSV enhancer and promoter were clearly functioning in Syrian hamster cells containing only a single copy of the plasmid. These cells grew efficiently in agar and were highly tumorigenic.

In this report we used 10W cells (passage 14), which is an immortal cell line isolated after treatment of SHE cells with asbestos (8, 9). We present evidence that these cells cotransfected with pSV2-neo and RSV DNAs, contained multiple copies of the RSV genome, but failed to express v-src RNA or pp60^c. This offers a cellular system to study the regulation of the src gene. Most of these clones were not morphologically transformed and failed to grow in soft agar. Three neo(RSV) clones were tumorigenic, and the tumor-derived cell lines from these clones expressed high levels of v-src RNA and protein. This increased expression was associated with an approximate 10-fold amplification in RSV DNA sequences and the appearance of double minute chromosomes. Our results suggest that the process of gene amplification alters the expression of the v-src gene in these cells.

MATERIALS AND METHODS

Cells and Transfection Assays. The cells used for study were 10W cells (passage 14), which is an immortal cell line isolated after treatment of SHE cells with asbestos (8, 9). DNA transfections were performed by the calcium phosphate precipitate technique (10, 11). The cells were plated at 1 x 10^5 cells/100-mm dish. The next day, the cells were cotransfected with 0.1 μg of pSV2-neo DNA and 5 μg of calf thymus DNA with or without 1 μg of RSV DNA. After 6 h, the cells were refed with fresh medium. Two days later, the transfected cells were split in a ratio of 1:3 with growth medium containing the antibiotic G418 sulfate (GIBCO Laboratories, Grand Island, NY) at 800 μg/ml (neo selection). After selection, colonies were encircled with cloning cylinders and drug-resistant colonies were trypsinized and isolated. These were then maintained in growth medium containing 100 μg of G418 per ml. Colonies were grown to approximately 10^5 cells and analyzed for DNA, RNA, and growth in 0.3% agar as described (5). At the same time, cells were injected s.c. into nude mice [nu/nu (BALB); Frederick Cancer Research Center, Frederick, MD] at 2 x 10^5 cells per site.

Recombinant Plasmid DNAs. Plasmid pSV2-neo (12) which contains the resistance gene to the antibiotic G418 was obtained from Steve Harris (National Institute of Environmental Health Sciences). Plasmid pJD300 which contains a genomic clone of the Prague A strain of RSV was obtained from J. T. Parsons (University of Virginia, Charlottesville, VA). This was derived from plasmid pJD100 (13) and contains Clal sites flanking the RSV genome. The 2.0-kilobase XhoI-EcoRI fragment from plasmid pRI-src (14) was nick translated and used as a src-specific probe.

DNA, RNA, and Protein Analyses. Preparation of high molecular weight DNA from cells, agarose gel electrophoresis, cytoplasmic dot blot analysis, probe preparation, and hybridization conditions were performed as described previously (5). Metabolic labeling of cells, immunoprecipitation with anti-pp60^c serum (15), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been previously described (16).

Chromosome Analyses and in Situ Hybridization. Cells in culture were prepared for chromosome analyses as described (17). A total of 50 metaphases were examined for chromosome number, aberrations, and double minute chromosomes following Giemsa staining. Five to 15 Q-banded metaphases were fully analyzed for karyotype (18). For use as a probe for in situ hybridization with metaphase or interphase cells, plasmid DNA containing the v-src gene was labeled by nick translation with [3H]dCTP (106 Ci/mmol), [3H]dATP (52 Ci/mmol), and [3H]dGTP (43 Ci/mmol) (New England Nuclear). Specific activities of 4 x 10^5-9 x 10^5 cpm/μg were obtained. The hybridizations were performed by the method described by Neel et al. (19). The slides were coated with NTB2 nuclear track emulsion (Kodak) and were exposed for 2 weeks at 4°C in a light-proof box. The exposed slides were developed in Kodak D-19 developer for 4 min, fixed, and rinsed in tap water. The slides were stained with Wright’s Giemsa solution.

Received 8/11/86; revised 5/7/87; accepted 6/4/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed.

2 The abbreviations used are: RSV, Rous sarcoma virus; SHE, Syrian hamster embryo; DM, double minute.

4663

Downloaded from cancerres.aacrjournals.org on June 9, 2017. © 1987 American Association for Cancer Research.
RESULTS

Transfection of 10W Cells with RSV DNA. We cotransfected 10W cells with pSV2-neo DNA (12) and a 10-fold excess of a genomic clone of RSV using the calcium phosphate precipitate technique (10, 11). Neo<sup>+</sup> cells were selected for G418 resistance, and individual neo<sup>+</sup> clones were isolated and tested for tumorigenicity and anchorage-independent growth. None of the 10 control neo<sup>+</sup> clones isolated after transfection with pSV2-neo DNA alone (neo<sup>+</sup> 10W clones) grew in soft agar (frequency, <10<sup>−6</sup>) or formed tumors in nude mice in 20 weeks. Out of 12 neo<sup>+</sup> clones isolated after cotransfection with pSV2-neo and RSV DNAs (neo<sup>+</sup> RSV 10W cell clones), only clone 61 grew in soft agar (frequency, 2 × 10<sup>−4</sup>), and 3 clones (41, 61, and 62) were tumorigenic with latency periods of 3 weeks. The soft agar-derived cell line from clone 61 was designated 61S, and tumor-derived cell lines from clones 41, 61, and 62 were designated 41T, 61T, and 62T, respectively.

Analyses of v-src DNA in neo<sup>+</sup> 10W Cell Clones and Tumor-derived Cell Lines. Cellular DNAs were extracted from one control neo<sup>+</sup> 10W cell clone, seven neo<sup>+</sup> RSV 10W cell clones, one soft agar-derived cell clone, and three tumor-derived cell lines. These DNAs were digested with EcoRI restriction endonuclease and were examined for the presence of v-src gene sequences by Southern blot analysis, using a src-specific probe. As shown in Fig. 1, all but one of the RSV-cotransfected cell clones contained the 3.0-kilobase v-src fragment containing the complete v-src gene coding sequences. The additional bands correspond to transfected v-src sequences which integrated between the EcoRI sites and created new plasmid-host cell junction fragments. Densitometric analysis of the 3.0-kilobase fragment from the Southern blot of cellular DNAs and known amounts of RSV DNA indicated v-src gene copies of approximately 6 (clone 41), 3 (clone 61), 8 (clone 62), and 10 (clone 32). These copy numbers were increased 10-fold in the soft agar-derived and tumor-derived cell lines when compared to an equal quantity of parental cell clone DNA. Four additional soft agar-derived clones from clone 61 contained amplified DNA copies. Longer exposure of the autoradiogram showed that each of the bands present in the amplified cell lines was also present in the parental cell clones. The cells retained the amplified RSV genomes after multiple passages (>50 population doublings). Restriction enzyme analyses of HpaI-digested DNAs also showed that there were no detectable rearrangements of RSV-integrated sequences during amplification. The restriction enzyme PvuI which cuts in the long terminal repeat of RSV released multiple fragments including a 10-kilobase fragment indicating the presence of intact copies of the RSV genome. The cellular DNAs were also examined for the presence of pSV2-neo DNA sequences by Southern blot analysis (data not shown). Amplification of the pSV2-neo sequences was observed in two out of three tumor-derived cell line DNAs when compared to the corresponding parental cell clone DNA.

Expression of pp60<sup>src</sup> in neo<sup>+</sup> 10W Cell Clones and Tumor-derived Cell Lines. To investigate whether the v-src gene sequences were being expressed in the parental neo<sup>+</sup> RSV 10W cell clones and whether this expression was increased in the cells that had amplified v-src sequences, we measured levels of pp60<sup>src</sup> protein. Three neo<sup>+</sup> RSV cell clones (41, 61, and 62) and three corresponding tumor-derived cell lines (41T, 61T, and 62T) were metabolically labeled with [35S]methionine; cellular extracts were prepared and immunoprecipitated with anti-pp60<sup>src</sup> serum and analyzed by SDS-PAGE (Fig. 2). The 60K band was present in all cells and corresponds to the endogenous hamster pp60<sup>src</sup> which was precipitated by this serum in addition to the Prague A pp60<sup>src</sup>. The results show that the three RSV parental cell clones (41, 61, and 62) and a control neo<sup>+</sup> 10W cell clone (clone 21) had little or no expression of pp60<sup>src</sup>, whereas the tumor-derived cell lines (41T, 61T and 62T) had elevated levels of pp60<sup>src</sup> (0.10 to 0.20% of the total labeled soluble protein). Clones 22, 32, and 72 did not express pp60<sup>src</sup>.

RNA Expression of the v-src Transfected Cell Sequences. To determine whether the reduction of pp60<sup>src</sup> expression in the parental neo<sup>+</sup> RSV 10W cell clones was due to lack of transcription of the v-src gene or to posttranscriptional defects, we analyzed RNA expression by cytoplasmic dot blot hybridization to a src-specific probe. Fig. 3 shows the dot blot analysis of cytoplasmic extracts isolated from the three neo<sup>+</sup> RSV cell clones (41, 61, and 62), the three corresponding tumor-derived cell lines (41T, 61T, and 62T) and one soft agar-derived cell clone.
equivalents of each cell clone and dilutions (1:2, 1:4, and 1:8) were applied to nitrocellulose filters and hybridized with \( ^{32}\)P-labeled v-jrc probe.

plasmic extracts were obtained from a control neo* 10W cell clone (21), neo* double minute chromosomes following Giemsa staining. Five to 15 Q-banded total of 50 metaphases were examined for chromosome number, aberrations, and RSV 10W cell clones (41, 61, and 62), soft agar-derived cell clones (0/5), or sequences were not amplified (41, 61, and 62). Four additional sequences were amplified (4IT, 6IT, 62T, and 6IS) but were cell lines and the soft agar-derived cell clone in which \(-\)src gene (6IS). The levels of \(-\)src RNA were high in the tumor-derived metaphases were fully analyzed for karyotype (18).* Numbers in parentheses, range.

Table 1 Double minute chromosomes in neo* RSV 10W cell lines

<table>
<thead>
<tr>
<th>Clones</th>
<th>% Metaphases* with DMs</th>
<th>Average number of DMs/metaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>neo 21</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>41</td>
<td>16%</td>
<td>6 (0–30)*</td>
</tr>
<tr>
<td>41T</td>
<td>98%</td>
<td>36 (0–100)</td>
</tr>
<tr>
<td>61</td>
<td>36%</td>
<td>36 (0–90)*</td>
</tr>
<tr>
<td>61S</td>
<td>48%</td>
<td>24 (0–50)</td>
</tr>
<tr>
<td>61T</td>
<td>82%</td>
<td>32 (0–100)</td>
</tr>
<tr>
<td>62</td>
<td>8%</td>
<td>3 (0–5)</td>
</tr>
<tr>
<td>62T</td>
<td>100%</td>
<td>31 (5–100)</td>
</tr>
</tbody>
</table>

* Cells in culture were prepared for chromosome analyses as described (17). A total of 50 metaphases were examined for chromosome number, aberrations, and double minute chromosomes following Giemsa staining. Five to 15 Q-banded metaphases were fully analyzed for karyotype (18).

Fig. 3. Dot blot analysis of v-src RNA in neo\(^*\) RSV 10W cell clones. Cytoplasmic extracts were obtained from a control neo\(^*\) 10W cell clone (21), neo\(^*\) RSV 10W cell clones (41, 61, and 62), soft agar-derived cell clones (61S), or tumor-derived cell lines (41T, 61T, and 62T). Aliquots (1) from 2 \( \times 10^6 \) cell equivalents of each cell clone and dilutions (1/2, 1/4, and 1/8) were applied to nitrocellulose filters and hybridized with \( ^{32}\)P-labeled v-src probe.

Table 1 Double minute chromosomes in neo\(^*\) RSV 10W cell lines

<table>
<thead>
<tr>
<th>Clones</th>
<th>% Metaphases* with DMs</th>
<th>Average number of DMs/metaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>neo 21</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>41</td>
<td>16%</td>
<td>6 (0–30)*</td>
</tr>
<tr>
<td>41T</td>
<td>98%</td>
<td>36 (0–100)</td>
</tr>
<tr>
<td>61</td>
<td>36%</td>
<td>36 (0–90)*</td>
</tr>
<tr>
<td>61S</td>
<td>48%</td>
<td>24 (0–50)</td>
</tr>
<tr>
<td>61T</td>
<td>82%</td>
<td>32 (0–100)</td>
</tr>
<tr>
<td>62</td>
<td>8%</td>
<td>3 (0–5)</td>
</tr>
<tr>
<td>62T</td>
<td>100%</td>
<td>31 (5–100)</td>
</tr>
</tbody>
</table>

* Cells in culture were prepared for chromosome analyses as described (17). A total of 50 metaphases were examined for chromosome number, aberrations, and double minute chromosomes following Giemsa staining. Five to 15 Q-banded metaphases were fully analyzed for karyotype (18).

The levels of v-src RNA were high in the tumor-derived cell lines and the soft agar-derived cell clone in which v-src gene sequences were amplified (41T, 61T, 62T, and 61S) but were undetectable in the parental clones in which v-src gene sequences were not amplified (41, 61, and 42). Four additional soft agar-derived clones from clone 62 were analyzed and also expressed high levels of v-src RNA. Northern blot RNA analysis indicated the major v-src mRNA produced in these cells was approximately 2.6 kilobases in length which is the size of spliced subgenomic RNA encoding v-src in virally infected cells. Non-tumorigenic neo\(^*\) RSV 10W cell clones 22, 32, and 72 were also negative for v-src RNA expression.

Presence of DM Chromosomes in Soft Agar and Tumor-Derived Cell Lines. Since many cell lines and human tumors which have amplified DNA sequences have been reported to contain DM chromosomes and/or homogeneous staining regions, we screened three parental neo\(^*\) RSV 10W cell clones (41, 61, and 62), the corresponding tumor-derived cell lines (41T, 61T, and 62T) and a soft agar-derived cell clone (61S) for the presence of DMs. Table 1 shows that a small percentage of the metaphases from the parental neo\(^*\) RSV cell clones contained DM chromosomes and the number of DMs per cell was low. In contrast, a high percentage of tumor-derived and soft agar-derived cells contained large numbers of DMs even following in vitro cultivation (Table 1). Fig. 4 shows a metaphase chromosome spread prepared from tumor-derived cells with DM chromosomes and a metaphase spread from parental cells which lacks DMs. No additional common chromosome changes or HSRs were apparent by Q-banded chromosome analyses of tumor-derived cells. The location of the amplified sequences in interphase cells could be detected by in situ hybridization (19) with a v-src-specific probe (data not shown). In tumor-derived cells the v-src sequences were amplified and localized in the nucleus, whereas in the RSV parental cells, reduced levels of v-src sequences were detected. The result from in situ autoradiography of a tumor-derived metaphase spread showed that the grains were distributed extrachromosomally, suggesting that the v-src copies were in the DMs. The autoradiographic grains were larger than the DMs precluding demonstration of a direct correspondence between the two.

**DISCUSSION**

We have investigated the expression of the v-src gene following cotransfection of the RSV genome and pSV2-neo DNA into an immortal SHE cell line, 10W. No expression of v-src RNA was detected in six parental neo\(^*\) RSV 10W cell clones even though the cells contained multiple copies of the v-src gene. However, three of these clones were able to form tumors in nude mice, and one clone was able to grow in soft agar, but only at a low frequency. In each case, authentic v-src RNA and protein expression were accompanied by a 10-fold amplification of v-src gene sequences in the tumor-derived and soft agar-derived cell lines. These cells also contained a larger number of DM chromosomes which correlated with the increased gene copy number. We interpret these results to indicate that the src gene was initially not expressed in these cells but, following gene amplification, expression resulted. The cells expressing pp60\(^*\) were able to grow in agar or in animals, and under these conditions selection of the transformed cells occurred.

In a previous paper, we reported that neoplastic transformation of tertiary passage SHE cells following transfection by RSV DNA was a multistep process (5). We suggested that this could be explained by an initial suppression of v-src gene expression by cellular factors and the loss of suppressor function when the cells became tumorigenic. Initial suppression of v-src gene sequences following DNA transfection of one immortal SHE cell line, 10W, also occurs. This is in contrast to our reported results with another carcinogen-induced immortal cell line which was efficiently transformed by RSV DNA (5). Suppression of transfected DNA is not a general property of 10W cells. Nine out of nine neo\(^*\) v-Ha-ras 10W cell clones isolated after cotransfection of pSV2-neo and Harvey murine sarcoma virus DNAs expressed v-Ha-ras RNA and p21\(^*\) in large amounts, were able to grow in soft agar with frequencies of \( \geq 1\% \), and were tumorigenic in nude mice with latency periods \( \leq 2 \) weeks. No amplification of v-Ha-ras gene sequences was detected.

The reason for the lack of RSV transcription in the 10W cells is not known; however, several mechanisms have been proposed to explain the regulation of RSV gene expression. Searle et al. (20) have described RSV-infected rat cell lines in which increased cytosine methylation of CCGG sequences near and within the src gene correlates with RSV transcriptional activity. RSV DNA cytosine methylation patterns were similar when HpaI/MspI and Smal restriction digests were compared between the parental neo\(^*\) RSV 10W cell clones and soft agar-derived or tumor-derived cell lines. Furthermore, attempts to relieve the suppression of RSV transcription by treatment of neo\(^*\) RSV 10W cell clones with 5-azacytidine have been negative to date (data not shown). Thus methylation does not appear to be inhibiting transcription.

\(^3\) L. Annab and T. Gilmer, unpublished data.
V-src AMPLIFICATION WITH TUMORIGENIC PHENOTYPE

Fig. 4. Metaphase chromosome spreads of neo<sup>+</sup> RSV 10W cell clone and tumor-derived cell line. A, clone 62 cell; B, tumor-derived clone 62 cell (62T) showing double minute chromosomes.

The mechanism involved in overcoming this suppression appears to include gene amplification of v-src sequences. The increased copy number may be important, although the actual number of genomes required is unclear. For example, clone 32 which contains 10 copies of the RSV genome, is nontumorigenic; whereas clone 61T, which contains 30 copies, is a tumor-derived cell line. Our results are consistent with a model involving amplification of v-src sequences and titration of a cellular repressor factor which allows transcription of the gene. Alternatively, the amplification process may introduce a mutation in the RSV genome or in cellular sequences flanking the genome which overcomes the suppression. The gene amplification could relieve a position effect regulating the transcription of the v-src gene at the initial site of integration of the RSV DNA. This mechanism has been suggested by Glanville (21) who reported an amplification of the v-src gene in an NIH 3T3 cell clone expressing v-src only at confluence. Gillespie et al. (22) also found that rearrangements of RSV proviral DNA in transformed rat cell clones were associated with regulation of RSV expression. We cannot detect any rearrangement of the v-src gene in the tumor-derived cell lines by Southern blot analyses; however, this method is limited and cannot examine the structure of the DM chromosomes. We are currently investigating the role of chromatin structure of the integrated RSV DNA which has also been shown to affect RSV transcription (23) and the factors that may interact with this DNA to regulate gene transcription.

ACKNOWLEDGMENTS

We are grateful to Lois Annab for providing excellent technical assistance and to Alma Gonzalez and Helena Bonner for typing the manuscript.

REFERENCES

Correlation of V-src Gene Amplification with the Tumorigenic Phenotype in a Syrian Hamster Embryo Cell Line

Tona M. Gilmer, Pattie W. Lamb, Mitsuo Oshimura, et al.

Cancer Res 1987;47:4663-4666.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/17/4663

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.