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# Unstable Expression and Amplification of a Transfected Oncogene in Confluent and Subconfluent Cells†

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**NIH 3T3 cells were transfected with a plasmid containing the transforming gene, v-*src*, from Rous sarcoma virus. One of the transformed cell lines isolated reverted to a flat, nontransformed morphology after cloning through soft agar. This cell line did not express the *src* gene and could no longer grow in soft agar. When these cells were held at confluence, spontaneous foci appeared which eventually covered the dish. The appearance of foci correlated with an increase in v-*src* gene expression, ability to grow in soft agar, and tumorigenicity in mice. When these transformed cells were trypsinized and held at subconfluence, both v-*src* expression and the transformed phenotype were progressively lost. Whereas rearrangement of the transfected gene was not detected, the gene copy number in the transformed cells was markedly increased (>50-fold). Confluence-dependent gene amplification and deamplification have been retained after several cycles of growth alternately at high and low density, in cells recloned through soft agar, and after cells had been maintained continuously at high or low density. The results suggest that, in this cell line, reversible gene amplification plays a central role in expression of the transfected gene.**

Transformed cells are distinguished from their nontransformed counterparts by differences in morphology, metabolism, and, perhaps most importantly, growth properties. Many cell lines require a solid substrate for growth but, when transformed, acquire the capacity for anchorage-independent growth. Further, although most cells show a density-dependent inhibition of growth, after transformation cells no longer become quiescent at confluence, but continue to grow (18, 31, 32, 47).

Work with temperature-sensitive mutants shows that, at least for the v-*src* oncogene, the transformed phenotype is dependent on continuous expression of the gene and can be completely reversed (18). Studies with partial transformation-defective mutants reveal dissociation of some parameters which are characteristically altered in the transformed cell. For instance, loss of surface fibronectin can be much reduced without loss of the ability to grow in soft agar (45). Due to the pleiotropic effects of the v-*src* gene product on the cell (5, 18), it is difficult to establish the relative importance of these parameters in transformation, using these mutants. Further, although the v-*src* gene originated by transduction of the endogenous c-*src* gene and shows considerable homology with it (41), recent work demonstrates that levels of c-*src* expression well above transforming levels of v-*src* do not result in neoplastic changes in mammalian cells (30) or cause transformation of chicken embryo fibroblasts (19). Whether the difference is due to the scattered single amino acid changes or the block of sequence divergence at the C terminus of the two proteins is not known (19), but it is clear that there is a qualitative difference between the two gene products.

In an attempt to shed light on the possible dose dependency of transformation by the v-*src* gene and the interrelationships between transformation parameters, the v-*src* structural gene was fused to a regulatable promoter from the mouse metallothionein-I (MT-I) gene. The MT-src plasmid, which was constructed by R. D. Palmiter (University of Washington, Seattle), was transfected into NIH 3T3 cells. Of the cell lines generated in this way, one appears to have a unique and unexpected phenotype: in this cell line expression of the transfected gene is independent of added zinc (an inducer of metallothionein) but is sensitive to cell confluence. Moreover, increased expression is associated with amplification of the gene. Both expression and amplification can be reversed by maintaining the cells at subconfluence. The results, which are described below, suggest that amplification plays a central role in the expression of the transfected gene.

## MATERIALS AND METHODS

**Cells, transfection, and isolation of cell lines.** NIH 3T3 cells were the gift of D. R. Lowy (National Institutes of Health, Bethesda, Md.). They were grown in Dulbecco modified Eagle medium with 7.5% calf serum. Transfection was according to standard protocols (16, 29). A total of $5 \times 10^5$ cells were plated per 6-cm dish. The next day 0.1 μg of plasmid and 20 μg of salmon sperm carrier DNA were calcium phosphate precipitated onto each recipient culture. After 20 min at room temperature, medium was added and the cells were incubated for 4 h at 37°C. The cells were then shocked with 15% glycerol for 4 min, washed, and incubated with new medium. One day after transfection 70 μM zinc acetate was included in the medium of some dishes. After 2 to 3 weeks the foci of transformation were picked, expanded, and cloned through soft agar (with or without added zinc acetate). Individual colonies were again picked, expanded, and characterized. For retransformation the flat cell lines were maintained at confluence, and the medium was changed every second day.

The efficiency of transformation with the MT-src plasmid was about 30 foci per μg of insert DNA in the absence of added zinc acetate and 110 foci per μg of insert DNA in its presence. For comparison, $10^3$ foci per μg of insert DNA were obtained with a clone of Harvey murine sarcoma virus (clone H-1 [13], the generous gift of D. R. Lowy) regardless of the presence or absence of exogenous zinc acetate.

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For high-density growth, cells were allowed to overgrow ($>2 \times 10^5$ cells per $cm^2$) before repassaging. For low-density growth, the cells were repassaged either before reaching confluence (at about $5 \times 10^4$ cells per $cm^2$) or before any morphological changes (rounding up, focus formation, or overgrowth) were detected at confluence.

N-1 cells were derived from NIH 3T3 cells by infection with Rous sarcoma virus Schmidt-Ruppin D (RSV SR-D) (a gift from L. R. Rohrschneider, Hutchinson Cancer Research Center) and cloning through soft agar. Fusion with chicken embryo fibroblasts showed that they contained rescuable transforming virus.

**MT-*src* gene fusion.** The construct used was the gift of R. D. Palmiter (University of Washington, Seattle). Briefly, the MT-I promoter fragment containing the metal responsive region, TATA homology, and cap site for transcription, but no translation start codon (about 1.8 kilobases [kb], spanned by *Eco*RI [5'] and *Bgl*II [3']) (12, 14), was fused to the v-*src* structural gene from RSV (strain Pr-C) which lacks promoter signals but has a translation start codon and a polyadenylation signal (about 2.2 kb, spanned by *Xho*I [5'] and extending to a linker-generated site in the U5 region [37]). A unique *Bam*HI site was generated at the junction of the MT and v-*src* sequences. Essentially the whole fused gene insert could be excised from pBR322 with *Eco*RI.

**Characterization of cell lines.** To characterize the changes correlating with altered morphology, the confluence-sensitive cell line was maintained in two different states after cloning through soft agar. The "nontransformed" cells were flat and were maintained at subconfluence. These cells provided the base line against which changes were compared. Transformation was obtained by maintaining these cells at confluence. The transformed cells thus represent a mass culture and were either analyzed without repassaging or maintained at high density for several passages before analysis. Transformed cells were also maintained at subconfluence for some experiments ("untransformed") but were not retransformed in the work described here.

*src*-specific kinase activity in the transfected cell lines was assayed according to Rohrschneider et al. (33), using tumor-bearing rabbit serum (a gift from L. R. Rohrschneider). Samples of lysates containing equivalent amounts of protein were used to allow direct comparison of samples within a single assay. Proteins were analyzed on discontinuous acrylamide gels (22).

For analysis by northern blotting, total cellular RNA was prepared by the guanidinium isothiocyanate method; polyadenylated RNA was selected over an oligodeoxythymidylate column. Electrophoresis was on 1.2% agarose formaldehyde gels, and the RNA was transferred onto nitrocellulose paper (23). Relative mRNA levels were determined by cytoplasmic dot hybridization (48).

Southern blot analysis was as described previously (23). The DNA concentration in samples to be analyzed was determined by spectrophotometry and, after restriction endonuclease digestion, by fluorescence (21), before electrophoresis on 1% agarose gels. Digestion with restriction enzymes was under the conditions described before (27). DNA dot blots were prepared essentially as described previously (20). Prehybridization and hybridization were performed as described before (23).

**Probes.** The "general *src*" probe was the 2.2-kb fragment derived by *Bam*HI and *Eco*RI digestion of the MT-src construct. This probe hybridizes with both the c-*src* and v-*src* genes and their products. The "v-*src*-specific" probe was the 580-base pair, 3'-terminal fragment derived from the general *src* probe by *Pst*I digestion. The gel-purified DNA fragments were nick translated to a specific activity of about $10^7$ cpm per μg.

**Growth in soft agar.** Cells were trypsinized, counted, and dispersed as a single cell suspension in 0.36% agar made up in Dulbecco modified Eagle medium with 7.5% calf serum, with or without 70 μM zinc acetate, over a 0.6% agar layer in the same medium. Fresh agar overlay was added to the dishes twice a week.

**Tumorigenicity.** BALB/c mice were injected intraperitoneally with $10^6$ cells and monitored for tumors. Positives were sacrificed at 2 to 3 weeks. Negatives were sacrificed at 8 weeks and examined by dissection. Tumor tissue was submitted for histological examination by a pathologist.

**Metaphase chromosome spreads.** Cells were treated with 0.075 μg of colcemid per ml for 12 h, selected by mitotic shake-off, and fixed with acid spread, and the DNA was stained with 4',6-dianidino-2-phenylindole.

## RESULTS

**Cell morphology and *src* kinase activity.** To examine quantitative aspects of transformation, we transfected NIH 3T3 cells with a plasmid containing the regulatable promoter from the mouse metallothionein-I gene (11) fused to the structural v-*src* gene from RSV (designated MT-src). Forty cell lines were picked from foci of transformation and cloned through soft agar. After expansion, two of these cloned cell lines were found to have reverted to a flat, nontransformed morphology. The altered morphology suggested that the transfected gene had been lost or inactivated, since the v-*src* gene is known to transform NIH 3T3 cells (5, 10).

Previous reports show that expression of transfected genes may be lost in the absence of selective pressure (3, 15, 34). The cell lines which had lost the transformed morphology were therefore maintained at confluence, thereby reproducing the selective pressure for focus formation used in the initial transfection assay. One cell line remained untransformed; the other formed spontaneous foci which eventually covered the whole dish. The changes undergone by this cell line are illustrated in Fig. 1. There is a clear switch from a morphology resembling the nontransformed parental cells (cf. Fig. 1A, B, and E) to a rounded morphology (Fig. 1C, D, and F) similar to that shown by NIH 3T3 cells infected with RSV SR-D and cloned through soft agar (Fig. 1G; N-1 cells).

To determine whether the change in morphology observed in this cell line correlated with a change in the expression of the *src* gene, a *src*-specific kinase assay was undertaken. This assay measures the ability of the *src* gene product to phosphorylate the immunoglobulin G heavy chain in an immune complex (8). Cells which had been maintained continuously at subconfluence after cloning through soft agar and showed a flat, nontransformed morphology (nontransformed cells) had low levels of kinase activity (Fig. 2a) very similar to those found in nontransformed NIH 3T3 cells (Fig. 2d). This low background level of activity is attributed to the endogenous c-*src* (33) and is presumably due to cross-reactivity of the antiserum with the endogenous mouse c-*src* gene product. By contrast, the cells which had been maintained at high density after focus formation and had a rounded, transformed morphology showed markedly elevated levels of immunoglobulin G heavy-chain phosphorylation (Fig. 2b). Further, when the transformed cells were trypsinized and maintained at subconfluent levels, the kinase activity was reduced to near-background levels (Fig. 2c).

The results suggested that, in this cell line, *src* kinase activity could be modulated by maintaining the cells at high

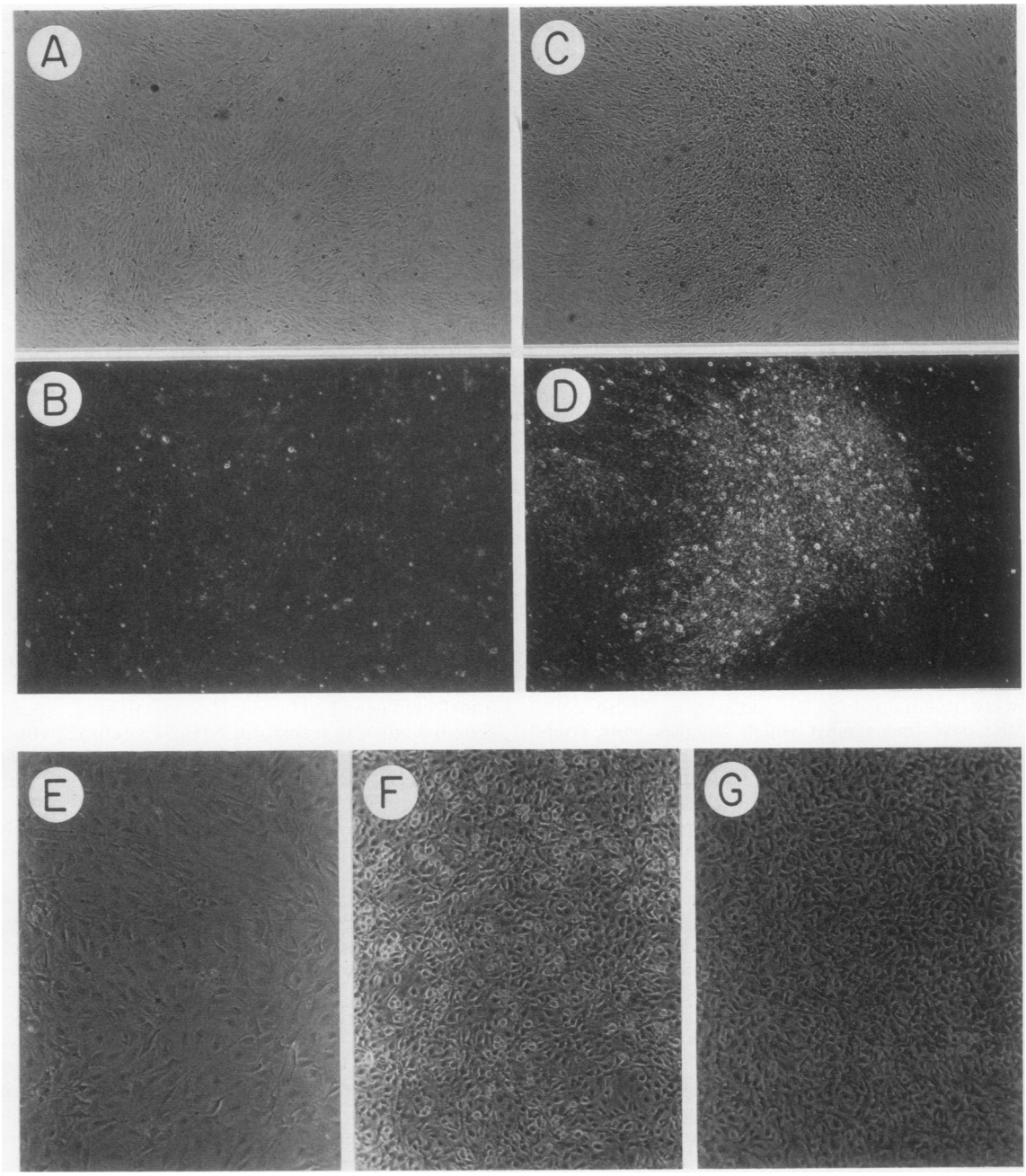


FIG. 1. Morphological changes undergone by cells containing transfected v-*src* at confluence. (A) Nontransformed cells held confluent for 2 weeks, showing flat, nontransformed morphology in an area without foci. (B) Same field as in (A) with darkground illumination. (C) Focus of transformed cells on the same dish as in (A). (D) Same field as in (C) with darkground illumination. (E) Parental NIH 3T3 cells held confluent for 2 weeks. No foci were detected. (F) Cells from the confluence-sensitive line shown in (A to D), maintained at high density for three passages. (G) NIH 3T3 cells infected with RSV SR-D and cloned through soft agar (N-1 cells).

or low density; i.e., the cell line is "confluence sensitive" for expression of the gene. Interestingly, although the transfected gene appeared intact (see below) and the metallothionein promoter responds to heavy metals in several contexts (6, 26), addition of zinc acetate to the culture medium did not affect the levels of *src* kinase in the confluence-sensitive cell line (Fig. 2) or their degree of transformation (Table 1). Control experiments reveal that this is not due to a failure of the cloned cells to respond to exogenous zinc, since the endogenous MT-I gene could be induced (data not shown).

**Kinetics of modulation of *src* kinase activity.** If focus formation in the confluence-sensitive cell line is attributable to *src* kinase activity, there should be a correlation between these two parameters. Subconfluent cells with flat morphology were therefore replated at high density and the appearance of foci was monitored. The cells were then lysed for the kinase assay. The first foci were visible after 3 days at confluence, when the kinase activity started to increase (Fig. 3A). Thereafter, both the number and size of the foci increased in apparent correlation with the kinase activity.

To examine the rate of loss of *src* kinase activity, morphologically transformed cells were replated at low density

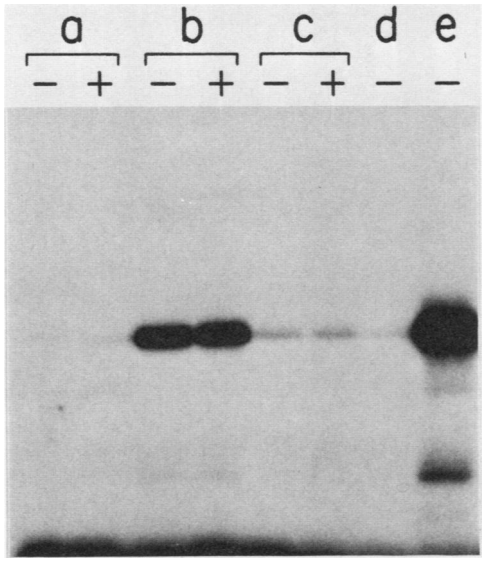


FIG. 2. *src* kinase activity in cells maintained at high or low density. Confluence-sensitive cells grown under different conditions were lysed, and samples containing equal amounts of protein were assayed for *src*-specific kinase activity. (a) Nontransformed cells showing a flat morphology, maintained at low density. (b) Cells with rounded (transformed) morphology maintained at high density. (c) Cells with partially rounded morphology, initially maintained at high density after focus formation and then passaged twice at low density. (d) Parental NIH 3T3 cells. (e) Chicken embryo fibroblasts infected with RSV Pr-C. −, No zinc added to medium; +, 70 μM zinc added to medium 48 h before harvest.

and the cells were lysed for kinase assay before reaching confluence. A progressive decline in the level of *src* kinase activity could be demonstrated (Fig. 3B). A clear difficulty with this experiment is the continuing growth of cells at subconfluence. This results in local variations in the cell concentration and is likely to affect the rate of loss of *src* kinase activity.

TABLE 1. Transformation and tumorigenicity of confluence-sensitive cells expressing v-*src*

| Cell line | *src* kinase activity[a] | Soft agar colonies | | Tumorigenicity in BALB/c mice[c] |
|---|---|---|---|---|
| | | Zn (−) | Zn (+)[b] | |
| Confluence sensitive | | | | |
| Nontransformed | − | 0 | 0 | 0/4 |
| Transformed | +++ | >2,800 | >3,000 | 4/4 |
| Partially untransformed | + | 350 | 500 | ND |
| Fully untransformed | − | 0 | 0 | 0/4 |
| NIH 3T3 | − | 0 | 0 | 0/4 |
| N-1 | ++++ | >50,000 | >50,000 | ND |
| Constitutive expressor of MT-src | ++ | >20,000 | >20,000 | 4/4 |

[a] *src* kinase activity represented on an arbitrary scale. (−) indicates level corresponding to parental NIH 3T3 cells.

[b] $10^5$ cells were dispersed in soft agar suspension on 60-mm dishes. The colony number was determined at 3 weeks. Zn (−), No added zinc; Zn (+), 70 μM zinc acetate included in all solutions.

[c] $10^6$ cells were injected intraperitoneally into BALB/c mice. At 2 to 3 weeks positives were sacrificed and dissected. Tumor tissue was diagnosed as malignant fibrosarcoma by histological examination. Negative mice showed no overt tumors upon dissection at 8 weeks.

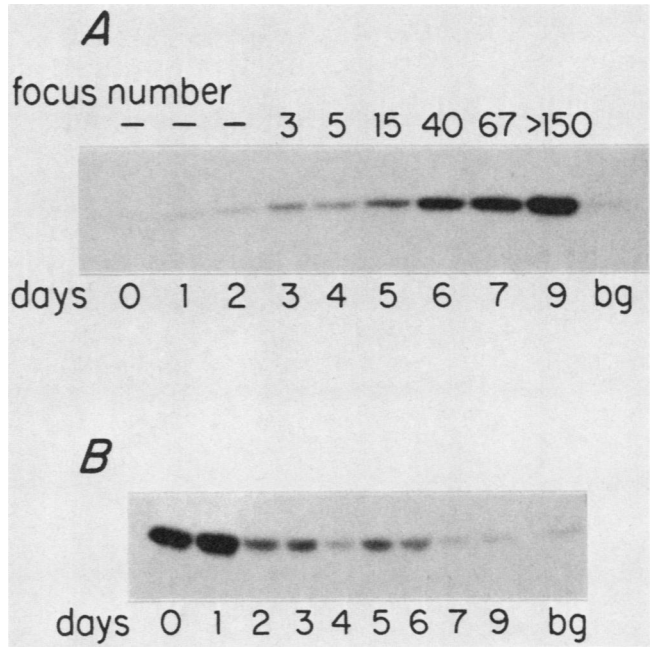


FIG. 3. Time course of modulation of *src*-specific kinase activity and transformed foci in confluence-sensitive cells. (A) Nontransformed confluence-sensitive cells maintained subconfluent and having flat morphology were replated. After incubation at confluence for the indicated number of days, the number of foci were determined (top numbers), the cells were lysed, and samples containing equal amounts of protein were assayed for *src* kinase activity. bg: Cells plated at low density and assayed at 5 days while still subconfluent. (B) Transformed cells maintained at high density and having rounded morphology were replated at low density. Cells were harvested, while still subconfluent, on the day indicated, and the *src* kinase activity was assayed in samples containing equivalent amounts of protein. bg: NIH 3T3 cells.

Although the kinetics varied in different experiments, the results support the notion that confluence affects the expression of *src* kinase activity, and this is reflected in the appearance of foci of transformation.

***src*-specific RNAs expressed at confluence.** The *src*-specific RNAs expressed at confluence were examined by northern blotting. Use was made of a v-*src*-specific probe to distinguish between transcripts from the transfected and the endogenous c-*src* genes. (This probe consisted of the last 40 bases of the coding region of v-*src*, where it diverges from c-*src* [41], and the U3 sequences derived from RSV.) Only one major v-*src*-specific RNA was detected in N-1 cells (Fig. 4A, lane d). Reprobing the same blot with the general *src* probe confirmed this result (Fig. 4B, lane d). The size of the authentic viral *src* mRNA is 2.6 kb (24, 37), in fairly good agreement with the size range indicated by the DNA markers (lanes M). The size expected for mRNA transcribed from the MT-src plasmid is about 2.3 kb, since the viral leader sequence (about 400 bases [17, 27]) has been replaced by about 60 bases from the MT-I message (14) and 70 bases 5′ to the splice acceptor site (the distance to the *Xho*I site used for the construction [37]; see Materials and Methods). No *src*-specific mRNAs were detected in the NIH 3T3 cells (lanes a) or in the nontransformed confluence-sensitive cells (lanes b). However, v-*src*-specific mRNA of the anticipated size was detected in the transformed cells (lanes c, arrow).

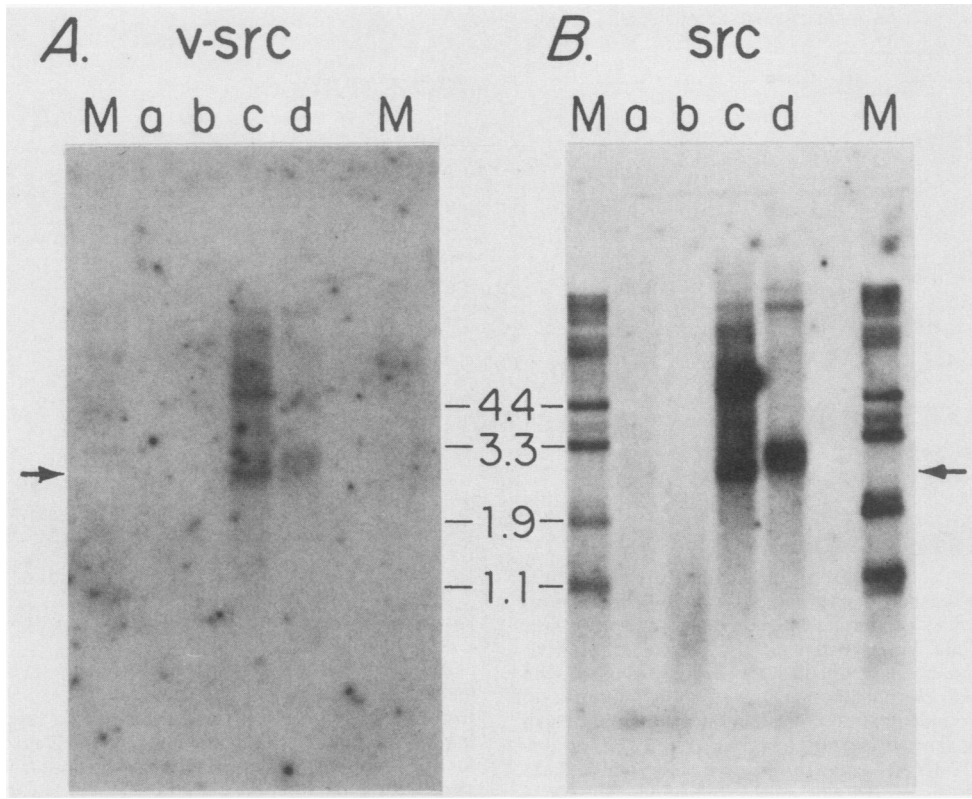


FIG. 4. Northern blot analysis of RNA from nontransformed and transformed confluence-sensitive cells. Total cellular RNA was prepared from nontransformed and transformed cells, and a sample was selected over an oligodeoxythymidylate column. The RNA was electrophoresed on agarose-formaldehyde gels and transferred to nitrocellulose. The northern blots were hybridized with the v-*src* and general *src* probes. (A) A 2-μg portion of oligodeoxythymidylate-selected RNA was analyzed, and the blot was probed with the v-*src*-specific probe. Lanes: a, NIH 3T3 cells; b, nontransformed confluence-sensitive cells; c, transformed confluence-sensitive cells; d, N-1 cells. (B) The blot shown in (A) was reprobed with the general *src* probe. M, pBR322 size markers (numbers indicate sizes in kilobase pairs). Arrow indicates position anticipated for MT-src mRNA.

Since this RNA comigrated with RNA from cells constitutively transformed by the MT-src plasmid (not shown), it most likely represents transcripts initiated at the MT-I promoter. This evidence suggests that the MT-src plasmid is correctly transcribed. However, other RNA species containing v-*src* as well as *src* sequences were expressed at confluence. In particular, an RNA migrating with a size of >5 kb was induced in amounts similar to the MT-src mRNA. Whether these RNAs arise from read-through transcription of host flanking sequences or promotion from a host promoter is not known. Immunoprecipitation data suggest that only the authentic $p60^{v\text{-}src}$ (identified by size and reactivity with two different antisera) was translated from the induced mRNAs (data not shown).

**Cells expressing v-*src* are transformed and tumorigenic.** To determine whether expression of v-*src* conferred a transformed phenotype as well as altered morphology on the confluence-sensitive cells, they were tested for the ability to grow in soft agar and cause tumors in mice. The nontransformed cells, which had background levels of *src* kinase activity, failed to grow in soft agar or cause tumors (Table 1). In contrast, cells with levels of *src* kinase activity near those found in virally infected NIH 3T3 cells (N-1 cells) gave rise to colonies (with an efficiency of about one-tenth that of the constitutively transformed cells) and caused tumors. The tumors were diagnosed as malignant fibrosarcomas, a pathology consistent with v-*src*-induced transformation (42). After being maintained at subconfluence, the cells ("partially untransformed" and "fully untransformed") lost the ability to grow in soft agar and cause tumors, along with *src* kinase activity. Thus expression of the v-*src* gene correlates well with the occurrence and loss of the transformed phenotype, and both can be fully reversed.

**The transfected gene is amplified in transformed cells.** The phenotype described has been retained after several cycles of alternating high- and low-density growth (>10 cycles), continuous passage at high or low density (for more than 30 passages), and recloning through soft agar (9 of 10 clones retained the confluence-sensitive phenotype). Further, when foci form, hundreds may be counted on a 60-mm tissue culture dish before they cover the dish. These observations argue for a stable integrated gene. Nonetheless, it was important to determine whether the fused gene was intact and if detectable changes in the gene occurred during induction.

To analyze the transfected gene, we digested DNAs from nontransformed and mass transformed cells (as opposed to an individual focus) with either endonuclease *Eco*RI or *Bam*HI. *Eco*RI should release the intact MT-*src* fused gene from the pBR322 sequences and genomic DNA, whereas *Bam*HI linearizes the plasmid and would release a fragment the full size of the input, if the plasmid is in tandem array. As shown in the Southern blot (Fig. 5A), a band comigrating with the fused gene was detected in the *Eco*RI digests of nontransformed and transformed cell DNAs, using the v-*src*-specific probe. Since this band also hybridized with a DNA

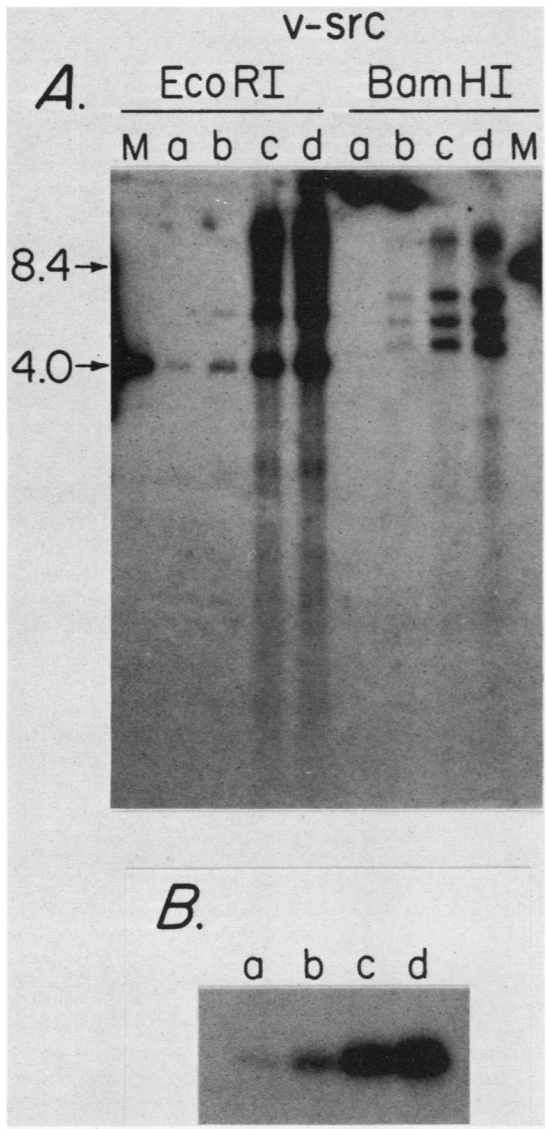


FIG. 5. Southern blot analysis of DNA from nontransformed and transformed confluence-sensitive cells. (A) DNA was prepared from cells at different degrees of transformation and digested with *Eco*RI or *Bam*HI, and 20 μg was electrophoresed on a 1% agarose gel. After transfer the nitrocellulose blot was probed for v-*src*-specific sequences. Lanes: a, nontransformed cells; b, partially transformed cells; c, transformed cells; d, transformed cells; M, input MT-src plasmid (25 pg) digested with *Eco*RI or *Bam*HI (numbers indicate sizes in kilobase pairs). (B) *src* kinase activity from dishes parallel to those used for preparation of DNA and analyzed in (A); lanes are as in (A).

fragment from the MT promoter but not with a pBR322 probe (not shown), it most likely represents the intact fused gene. The intensity of the hybridizing bands increased >50-fold in the induced cells. The additional bands (about 6 and 20 kb) which are detected in *Eco*RI-digested DNA from transformed cells are most likely junction fragments between host cell and transfected DNA since they contain new restriction sites and are not detected with the MT promoter probe. The control for expression of the amplified gene is shown in Fig. 5B; both gene amplification and *src* kinase activity appear to increase in parallel.

Alterations in the mobilities of the hybridizing bands during induction were not detected in the *Eco*RI or the *Bam*HI digests (Fig. 5A), suggesting that rearrangement was not occurring upon amplification. The latter digests also revealed that full-sized copies of the plasmid were not present. Because the MT-*src* fused gene appeared intact, sequences from pBR322 must have been deleted. The undigested DNAs were also analyzed by Southern blotting; the hybridizing material migrated at about 48 kb, with the bulk of the DNA (not shown). The transfected gene is therefore part of a high-molecular-weight complex and is most likely integrated. The intensity of the hybridizing bands appeared to increase coordinately in the induced cells (Fig. 5A). This suggests that they are part of one amplifying unit and is consistent with a single site of integration for the transfected gene. From the sizes of the bands detected with the v-*src* and pBR322 probes, the minimum size that can be estimated for this unit is about 40 kb.

**Amplification is reversible and parallels expression.** If amplification is important in regulating expression of the v-*src* gene, it should increase at confluence in parallel with expression and decrease at subconfluence. To test this, cells were maintained at confluence or subconfluence for various lengths of time. DNA and cytoplasmic extracts prepared from the same cells were then probed for *src* DNA and RNA sequences by dot hybridization, using the general *src* probe. In the experiment shown in Fig. 6A, amplification at confluence was rapid and was readily detected after 2 days. An apparently corresponding increase in cytoplasmic RNA was observed. Conversely, in the subconfluent cells, *src* gene sequences were lost, and a parallel loss of *src* cytoplasmic RNA was detected (Fig. 6B). As noted above for the kinetics of modulation of *src* kinase activity, the time courses of amplification and deamplification varied in different experiments. The results show that amplification is reversible and correlates fairly well with the levels of *src*-specific RNA, implying that amplification and expression are linked. It should be noted that the RNA dot blots in Fig. 6A and B were hybridized separately, using different preparations of probe, and cannot, therefore, be compared directly.

**DMs are found in transformed cells.** Gene amplification is often associated with the appearance of homogeneously staining regions or double minute chromosomes (DMs). Characterisitcally, unstable amplification is associated with the latter (7, 35, 40). Metaphase chromosome spreads were prepared from nontransformed and transformed cells which had been maintained at high density for several passages and stained for DNA. Figure 7 shows that multiple minute chromosomes, both single and double, could be detected in the transformed cells but not in the nontransformed cells; nor could they be detected in confluence-sensitive cells which had been transformed and then maintained at subconfluence, parental NIH 3T3 cells, in cells infected with virus (N-1 cells), or another cell line which constitutively expresses the transfected MT-*src* fused gene (data not shown).

## DISCUSSION

**Expression of the transfected gene and transformation.** In many respects the expression and biological activity of the v-*src* gene construct in the confluence-sensitive cell line were normal. *src*-specific kinase activity and v-*src* RNA sequences were detected in expressing cells (Fig. 2 and 4); these cells showed a transformed (rounded) morphology (Fig. 1), were able to grow in soft agar, and caused tumors in mice (Table 1) of the type expected for transformation by v-*src* (42). Moreover, when transformed cells were maintained at subconfluence until v-*src* kinase activity was unde-

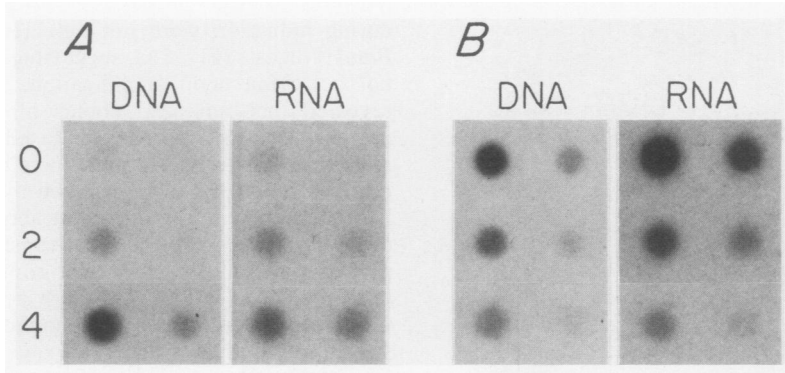


FIG. 6. *src* sequence amplification and expression in confluent and subconfluent cells. Nontransformed confluence-sensitive cells were maintained at confluence, and transformed induced cells were maintained at subconfluence for the indicated number of days. The cells were lysed for preparation of cytoplasmic extracts, and DNA was prepared from the pelleted nuclei. Amounts of 10 and 2 μg of DNA were applied to nitrocellulose, whereas cytoplasmic extracts from $2 \times 10^5$ cells and a twofold dilution were analyzed. The blots were probed with the general *src* probe. Because the cytoplasmic extract blots ("RNA," A and B) were hybridized separately and with different preparations of probe, the two blots are not directly comparable. (A) Nontransformed confluence-sensitive cells were maintained at confluence for the indicated number of days. (B) Transformed cells were maintained at subconfluence.

tectable, the transformed phenotype was no longer observed (Table 1). The correlation between expression of this known transforming gene and the transformed phenotype strongly suggests that the v-*src* gene product is responsible for the transformation observed.

**Selection versus induction.** The confluence-dependent nature of v-*src* expression in this cell line is unusual. Only 1 of 40 cell lines generated by transfection showed this phenotype. Although expression of the transfected gene was not detected in the nontransformed cells (Fig. 2 and 4; Table 1), there is the possibility that a small proportion of the cells spontaneously amplify v-*src* sequences or contain amplified v-*src* sequences and are constitutively transformed. At confluence these cells would be selected since they are not density inhibited and can overgrow the nontransformed cells (18, 31, 32, 46). The "induction" observed would then be the result of selection and not a true induction.

Several lines of evidence argue against this hypothesis. (i) The original cell line was cloned through soft agar, and so most likely originated from one cell. (ii) When recloned

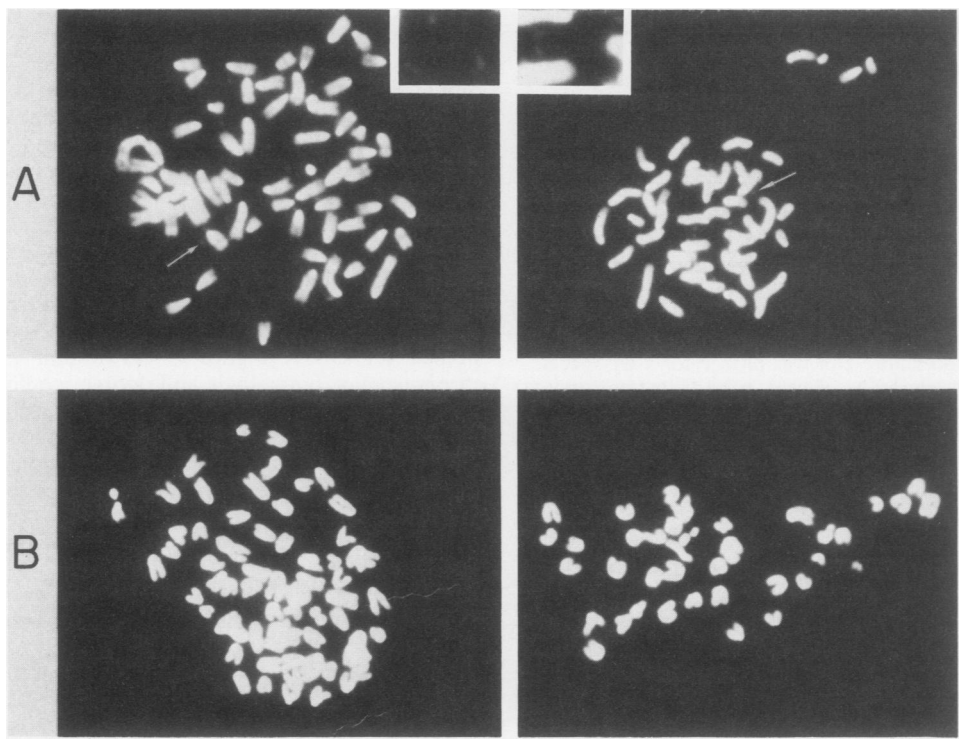


FIG. 7. Metaphase chromosome spreads from transformed and nontransformed confluence-sensitive cells. Cells transformed at confluence, and maintained at high density for several passages, were treated with colcemid and harvested by "mitotic shake-off." The fixed spread chromosomes were stained with 4',6-dianidino-2-phenylindole. Two spreads are shown from each sample. Inserts show enlargements of central areas containing DMs. (A) Transformed confluence-sensitive cells; (B) nontransformed confluence-sensitive cells. Arrows indicate examples of minute chromosomes.

through soft agar, the confluence-sensitive phenotype was retained at the level of gene amplification in 9 of 10 separate cell lines (not shown). (iii) Cells which have been continuously maintained at high or low density for several months (more than 30 passages), which should select for either transformed or nontransformed cells, are still confluence sensitive for transformation (not shown). (iv) Neither morphologically nontransformed cells nor cells which have been transformed and then maintained at low density grow in soft agar (Table 1). This is unexpected if part of the population is constitutively transformed or spontaneous amplification occurs at subconfluence. (v) The induced cells grow at low efficiency (3%) in soft agar compared with constitutively transformed cells (20 to 50%; Table 1). This observation is most readily rationalized in terms of deamplification at subconfluence since the cells are dispersed as a single cell suspension in soft agar. Only after some rounds of division would a self-sustaining clone, in which the cells are in close contact ("confluent"), be formed.

It therefore seems reasonable to conclude that the initial phase of v-*src* expression is due to induction in nonexpressing cells. However, once the cells have become transformed there will be selection because transformed cells are not density inhibited and can overgrow.

**Amplification and expression.** At confluence, the increased expression of the v-*src* gene parallels the increase in the gene copy number at the level of kinase activity (Fig. 5A and B) and cytoplasmic RNA (Fig. 6A). At subconfluence a similar correlation was observed, with both the gene copy number and RNA decreasing (Fig. 6B). Further, preliminary experiments indicate that inhibitors of DNA synthesis also prevent accumulation of v-*src* RNA at confluence and prevent its loss at subconfluence (unpublished data). Thus the amounts of gene template and transcripts appear to correlate. Since the cell line is apparently clonal, these data suggest that regulation of expression of the transfected gene is at the level of amplification.

Corroborative evidence for gene amplification comes from the detection of DMs in the transformed cells, but not the nontransformed cells (Fig. 7), or cells which have been maintained at high and then low density, or constitutively transformed cells. Although the gene has not been proven to be carried by these structures, and the time course of DM induction is not yet known, the generally unstable nature of the structures (7, 35, 40) fits well with the phenotype described for the confluence-sensitive cell line.

**Is the phenotype a position effect?** As revealed by Southern blotting, the transfected v-*src* gene fusion was intact and contained the metal responsive region of the MT-I promoter (Fig. 5A) (6, 26), and at a gross level no rearrangement of the gene was detected during amplification (Fig. 5A). Northern blotting showed that a v-*src* mRNA species of the size anticipated for transcription from the MT-I promoter was expressed at confluency (Fig. 4), suggesting that the intact copies of the gene could function as template. However, the response of the promoter to zinc was clearly lacking in this cell line (Fig. 2, Table 1). Because the transfected gene is most likely integrated, the simplest explanation for the lack of zinc response is a position effect due to the DNA sequences flanking the v-*src* gene, although mutation cannot be ruled out.

A position effect would also appear to provide an explanation for amplification of the v-*src* gene at confluence. The confluence-sensitive phenotype is unusual: neither virally infected cell lines (five have been surveyed) nor other lines derived by transfection of the MT-src plasmid show amplification or a change in the level of *src* expression at confluence (unpublished data). The apparent rarity of the phenotype would fit well with the nontargeted nature of the integration undergone by transfected genes (40). Further, there is precedent for the hypothesis, since in cell lines transfected with the cloned CAD gene and then subjected to selection, differences in the frequency of amplification of up to 100-fold were detected (44). This result has been interpreted as a position effect, because the sites of integration were all different. It thus seems clear that the site of integration can influence the ability of a gene to amplify and be expressed. Indeed, the developmentally specific amplification of the chorion genes of *Drosophila* spp. appears to be a position effect subject to both *cis* (38, 39) and *trans* regulation (28), the elevated levels of expression associated with amplification of these genes being required for viability of the eggs (28). Finally, work with the *dhfr* gene has shown that a twofold or greater amplification can occur within one cell cycle (25).

The hypothesis that amplification of the transfected v-*src* gene is a position effect has some interesting implications: the v-*src* gene has most likely integrated into a region close to an origin of replication. Moreover, if the locus amplifies at confluence in the absence of v-*src*, it raises the possibility that other genes in the amplifying unit may play a role in either maintaining or regulating the growth state of the cells. The high degree of amplification obtained in the presence of v-*src* should simplify cloning of the flanking sequences for examination of this hypothesis in detail.

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## LITERATURE CITED

1. **Alitalo, K., M. Schwab, C. C. Lin, H. E. Varmus, and J. M. Bishop.** 1983. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc. Natl. Acad. Sci. U.S.A. **80:** 1707–1711.
2. **Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253:**1357–1370.
3. **Bacchetti, S., and F. L. Graham.** 1977. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex virus DNA. Proc. Natl. Acad. Sci. U.S.A. **74:**1590–1594.
4. **Beach, L. R., and R. D. Palmiter.** 1981. Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. Proc. Natl. Acad. Sci. U.S.A. **78:**2110–2114.
5. **Bishop, J. M., and H. E. Varmus.** 1980. Functions and origins of retroviral transforming genes, p. 999–1108. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Brinster, R. L., H. Y. Chen, R. Warren, A. Sathy, and R. D. Palmiter.** 1982. Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. Nature (London) **296:**39–42.
7. **Brown, P. C., S. M. Beverley, and R. T. Schimke.** 1981.

Relationship of amplified dihydrofolate reductase to double minute chromosomes in unstably resistant mouse fibroblast cell lines. Mol. Cell. Biol. 1:1077–1083.
8. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. 75:2021–2024.
9. **Collins, S., and M. Groudine.** 1982. Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukemia cell line. Nature (London) 298:679–681.
10. **Copeland, N. G., A. D. Zelenetz, and G. M. Cooper.** 1980. Transformation by subgenomic fragments of Rous sarcoma virus. Cell 19:863–870.
11. **Durnam, D. M., and R. D. Palmiter.** 1982. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J. Biol. Chem. 256:5712–5716.
12. **Durnam, D. M., F. Perrin, F. Gannon, and R. D. Palmiter.** 1980. Isolation and characterisation of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. U.S.A. 77:6511–6515.
13. **Ellis, R. W., D. DeFeo, J. M. Maryal, H. A. Young, T. Y. Shih, E. H. Chang, D. R. Lowy, and E. M. Scolnick.** 1980. Dual evolutionary origin for the rat genetic sequence of Harvey murine sarcoma virus. J. Virol. 36:408–420.
14. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature (London) 292:267–269.
15. **Graf, L. H., G. Urlaub, and L. A. Chasin.** 1979. Transformation of the gene for hypoxanthine phosphoribosyltransferase. Somatic Cell Genet. 5:1031–1044.
16. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.
17. **Hackett, P. B., R. Swanstrom, H. E. Varmus, and J. M. Bishop.** 1982. The leader sequence of the subgenomic mRNAs of Rous sarcoma virus is approximately 390 nucleotides. J. Virol. 41:527–534.
18. **Hanafusa, H.** 1977. Cell transformation by RNA tumour viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Press, New York.
19. **Iba, H., Y. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 81:4424–4428.
20. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridisation procedure. Nucleic Acids Res. 7:1541–1552.
21. **Labarca, C., and K. Paigen.** 1980. A rapid simple and sensitive DNA assay procedure. Anal. Biochem. 102:344–351.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 227:680–685.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Mardon, G., and H. E. Varmus.** 1983. Frameshift and intragenic suppressor mutations in a Rous sarcoma provirus suggest *src* encodes two proteins. Cell 32:871–879.
25. **Mariani, B. D., and R. T. Schimke.** 1984. Gene amplification in a single cell cycle in Chinese hamster ovary cells. J. Biol. Chem. 259:1901–1910.
26. **Mayo, K. E., R. Warren, and R. D. Palmiter.** 1982. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. Cell 29:99–108.
27. **O'Farrell, P.** 1981. Replacement synthesis method of labelling DNA fragments. Focus 3:1–3.
28. **Orr, W., K. Komitopoulou, and F. C. Kafatos.** 1984. Mutants suppressing in *trans* chorion gene amplification in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 81:3773–3777.
29. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. 31:360–369.
30. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-*src* and chicken c-*src* in rat cells demonstrates qualitative differences between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. Cell 37:131–139.
31. **Perbal, B.** 1984. Transformation parameters expressed by tumour-virus transformed cells, p. 163–195. *In* G. Klein (ed.), Advances in viral oncology, vol. 4. Raven Press, New York.
32. **Pollack, R., S. Chen, S. Powers, and M. Verderame.** 1984. Transformation mechanisms at the cellular level, p. 3–28. *In* G. Klein (ed.), Advances in viral oncology, vol. 4. Raven Press, New York.
33. **Rohrschneider, L. R., R. N. Eisenman, and C. R. Leitch.** 1979. Identification of a Rous sarcoma virus transformation-related protein in normal avian and mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 76:4479–4483.
34. **Scangos, G. A., K. M. Huttner, D. K. Juricek, and F. H. Ruddle.** 1980. Deoxyribonucleic acid-mediated gene transfer in mammalian cells: molecular analysis of unstable transformants and their progression to stability. Mol. Cell. Biol. 1:111–120.
35. **Schimke, R. T.** 1982. Gene amplification, p. 317–339. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Schwab, M., K. Alitalo, H. E. Varmus, J. M. Bishop, and D. George.** 1983. A cellular oncogene (c-Ki-*ras*) is amplified, overexpressed and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature (London) 303:497–501.
37. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell 32:853–869.
38. **Spradling, A. C.** 1981. The organization and amplification of two chromosomal domains containing Drosophila chorion genes. Cell 27:193–201.
39. **Spradling, A. C., and A. P. Mahowald.** 1981. A chromosomal inversion alters the pattern of specific DNA replication in Drosophila follicle cells. Cell 127:203–209.
40. **Stark, G. R., and G. M. Wahl.** 1984. Gene amplification. Annu. Rev. Biochem. 53:447–491.
41. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell 32: 881–890.
42. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1980. Pathogenesis of retro-virus induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-apsartate-resistant hamster cells. J. Biol. Chem. 254:8679–8689.
44. **Wahl, G. M., B. Robert de Saint Vincent, and M. L. DeRose.** 1984. Effect of chromosomal position on amplification of transfected genes in animal cells. Nature (London) 307:516–520.
45. **Weber, M. J.** 1984. Malignant transformation by Rous sarcoma virus: from phosphorylation to the transformed phenotype, p. 249–268. *In* G. Klein (ed.), Advances in viral oncology, vol. 4. Raven Press, New York.
46. **Weiss, R.** 1970. Studies on the loss of growth inhibition in cells infected with Rous sarcoma virus. Int. J. Cancer 6:333–345.
47. **Weiss, R.** 1980. Experimental biology and assay of retroviruses, p. 209–260. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **White, B. A., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridisation. J. Biol. Chem. 257:8569–8572.