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# DNA methylation prevents the amplification of *TROP1*, a tumor-associated cell surface antigen gene

(DNA transfection/Trop-2/JM/JAR/BEWO)

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ABSTRACT **We tested the hypothesis that different genes can have different abilities to be amplified after transfection under comparable selection conditions. DNA from human lymphoid or choriocarcinoma cell lines was transfected into L cells. Transfectants for *CD5*, *CD8A*, *TROP1*, and *TROP2*, genes expressed on lymphocytes or trophoblast and carcinomas, were selected by fluorescence-activated cell sorting. To select for amplification of the transfected gene we cloned twice by fluorescence-activated cell sorting the transfectants with the highest expression. We analyzed a total of 38 families (1768 clones) derived from the original transfectants. We then analyzed by Southern blotting the clones with the highest increase in surface expression and determined the copy number of each transfected gene. *CD5*, *CD8A*, and *TROP2* were amplified with high frequency and progressively, whereas *TROP1* essentially was not amplified at all. We examined the hypothesis that DNA methylation prevents the amplification of the *TROP1* gene by treating JAR choriocarcinoma cells with 5-azacytidine to decrease DNA methylation. DNA extracted at different times after the treatment was used for transfection. When DNA that showed demethylation of the *TROP1* gene was used, 16 Trop-1 transfectants were obtained and 6 of them were found to contain up to 40 copies of the *TROP1* gene per haploid genome. Thus, we showed that transfectants obtained from a demethylated *TROP1* gene were amplified efficiently and progressively. We propose that DNA methylation affects DNA amplification either by altering the recognition of methylated DNA sequences or by changing the conformation of the chromatin of methylated segments. We speculate that DNA methylation is a determinant of gene amplification *in vivo*, for example in tumor cells.**

Gene amplification—i.e., an increase in gene copy number per genome—has been observed in several cell systems (1–7). Definition of the molecular mechanisms of gene amplification is of both theoretical and practical interest, since oncogenes and drug-resistance genes both undergo amplification (2, 5, 8).

Usually, selection for gene amplification is performed by killing cells that do not amplify using a cytotoxic drug that interferes with a metabolic pathway involving the gene of interest (2). Thus, it is difficult to study the amplification of genes that cannot confer a survival advantage and to identify genes incapable of amplification.

Thus, we chose to study gene amplification after transfection of genomic DNA and selection of transfectants by fluorescence-activated cell sorting (FACS) (9). This selection procedure is not based on cell killing and can be applied equally well to several different genes (10). Further advantages are that genes are amplified at high frequency after transfection (6) and can be tested for their methylation status and without disturbing their flanking sequences (10, 11). Using this approach, we investigated the amplification ability of several cell surface antigen genes [i.e., *CD5* (12), *CD8A* (13), *TROP1* (14–16), and *TROP2* (14, 17)] to identify genes that either could or could not be amplified. The genes above were chosen because they are human single-copy genes encoding single polypeptides and are efficiently transfected into mouse L cells (6, 10, 12, 13, 15–17). However, CD5, CD8$\alpha$, and Trop-2 show quite heterogeneous patterns of expression after transfection in average levels, ranges, and stability of expression (S.A., unpublished data). On the other hand, most Trop-1 transfectants show stable narrow ranges of surface expression, unlike most of the transfectants that do amplify their transfected genes. Thus, *TROP1* appeared as a good candidate for a gene that could not be amplified.

## MATERIAL AND METHODS

**Immunofluorescence.** Fluorescein-conjugated anti-Leu-1 (CD5) and anti-Leu-2a (CD8$\alpha$) antibodies were provided by Becton Dickinson. The anti-Trop-1 and anti-Trop-2 (14) antibodies were produced as described (18). Fluorescence analysis, sorts, and clonings were performed using a modified FACS II and a FACStar (Becton Dickinson) (9). To improve the detection of transfectants, subtraction of cell autofluorescence (19) and overcompensation in the red channel (20) were performed during sorting.

**DNA Transfection.** The calcium phosphate coprecipitation technique was followed as described (10) using guanidinium-purified (21) genomic DNA from the JM, BEWO, or JAR cell lines (6, 10, 22). Transfectants were selected for expression of CD5, CD8$\alpha$, Trop-1, and Trop-2, respectively, as described (6, 10, 13, 16, 17). Pure populations of transfected cells were obtained. From each population of transfectants, the 0.1% of the cells with the highest expression was cloned by single-cell sorting (first-round clones). At least 24 clones from each sort were reanalyzed. The two brightest clones from each group of first-round clones were cloned again, originating the second-round clones. Each independent transfectant together with the corresponding first- and second-round clones constituted a family of transfectants.

Transfectant names indicate how they were selected (Figs. 1–6). The source of the transfected DNA (JM, BEWO, or JAR) is indicated first, followed by the number identifying each transfection. The antigen selected (*leo*, CD5; *let*, CD8$\alpha$; *tro*, Trop-1; *trt*, Trop-2) comes next, followed by a period and the number of the first- and second-round clones, where appropriate. For example, JM14*tro*.4.17 is a clone obtained from L cells of plate 14 transfected with DNA from JM cells, selected for Trop-1 expression, cloned a first time (clone number 4), and recloned (clone number 17). For transfectants

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Abbreviations: FACS, fluorescence-activated cell sorting; PBL, peripheral blood lymphocyte.

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obtained with demethylated JAR DNA, aza indicates treatment with 5-azacytidine and is followed by the length of recovery in culture—i.e., 5 days (JARaza5D), 12 days (JARaza12D), or 6 weeks (JARaza6W). A slash followed by a number indicates the number of further batch sorts of each transfectant, where appropriate.

**DNA Hybridization.** Copy numbers of the transfected genes were determined by Southern blotting (23). Filters were hybridized with either CD5 (12), CD8α (13), KS1-4/Trop-1 (15), or Trop-2 (S.A. and M.N., unpublished data) probes. λ DNA cut with *Hin*dIII and *Eco*RI/*Hin*dIII was used as molecular weight standards.

## RESULTS

By transfecting DNA from JM cells, we produced 10 CD5, 13 CD8α, and 9 Trop-1, but no Trop-2, L-cell transfectants. However, 6 independent Trop-2 transfectants were readily obtained by transfecting DNA from the cell line BEWO, which expresses Trop-2 (22). JM cells do not express Trop-2, and we have previously shown that DNA methylation can prevent gene transfection from nonexpressing sources (10). Thus, differential methylation of the *TROP2* gene in JM and BEWO cells may explain these results.

From each transfectant we cloned the cells giving the 0.1% highest expression. The resulting first-round clones were analyzed, and the two brightest clones were cloned again, yielding second-round clones. Cloning was performed to avoid differential growth advantage of multiple independent transfectants from the same dish. Likely examples of multiplicity of transfection are shown in Fig. 4 (lanes 3–6 and 4–7). Selecting clones also minimized the effects of a possible differential growth advantage of cells expressing a transfected gene at different levels. A total of 1768 clones were analyzed by FACS (Table 1). Cell survival was 30% on average and was not statistically different between different groups of cells transfected for different genes or between cells expressing the transfected genes at different levels. Also, no significant differences were found between first- and second-round clones, despite higher average gene expression of the latter. The fluorescence distribution profiles of second-round clones were compared with the corresponding first-round clones and with the transfectants before cloning (Fig. 1 and Tables 1 and 2). Clear increases in surface expression were seen in 7 of 10 families of CD5 transfectants, 9 of 13 families of CD8α transfectants, and in 4 of 6 families of Trop-2 transfectants. In contrast, only marginal if significant increases were seen in 2 of 9 families of Trop-1 transfectants and in only 5.8% of the Trop-1-expressing clones. Unstable clones (i.e., clones with lower expression than the parental transfectant) were frequently found in a large fraction of the clone families analyzed (Table 1, column "<1×").

The clone families showing the greatest increase in surface expression during the selection procedure were analyzed by Southern blotting. As controls, Southern blots were also performed on clone families with no increase in surface expression (one for CD5, one for Trop-2, and two for Trop-1).

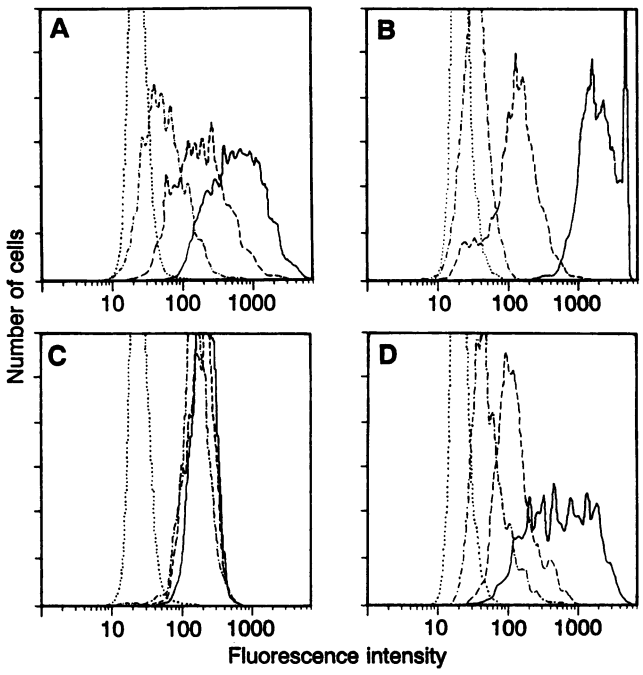


FIG. 1. Examples of FACS profiles of L cells selected for CD5 (*A*; JM10*leo*), CD8α (*B*; JM07*let*), Trop-1 (*C*; JM09*tro*), or Trop-2 (*D*; BEWO09*trt*). Dotted and dashed line, transfectants before cloning; dashed line, first-round clones; solid line, second-round clones; dotted line, unselected control L cells. Note that the Trop-1 transfectant does not change its pattern of expression over months in culture and multiple rounds of selection by FACS.

We detected significant gene amplification (i.e., a 3- to 40-fold increase in gene copy number) in three of five families of CD5 transfectants, in five of six families of CD8α, and in two of four families of Trop-2. Only a 2-fold increase in gene copy number was observed in two of four Trop-1 families analyzed (Table 3 and Figs. 2–5). Interestingly, the latter occurred in Trop-1 transfectants before cloning and remained stable over two successive rounds of cloning. For comparison, in all other transfectants, progressive increases in gene copy number were observed during the selection procedure (Table 3 and Figs. 2, 3, and 5). In the JM09*tro* family the hybridization signal was about one-half that of peripheral blood lymphocyte (PBL) DNA and remained stable during the selection procedure (Fig. 4*B*). This likely indicates the presence of a single copy of the *TROP1* gene per diploid genome.

Clones with lower gene copy number than that in the parental populations were observed in two of five families of CD5, in four of six families of CD8α, and in two of four families of Trop-2 transfectants. None was observed in Trop-1 transfectants. Interestingly, all clone families with cases of loss of the transfected genes also contained amplificants.

Table 1. Surface expression of the transfected genes in individual clones by FACS analysis

| Gene | Clones analyzed | Final level of surface expression after FACS selection* | | | | |
|---|---|---|---|---|---|---|
| | | <1× | 1× | 2–4× | 5–10× | 11–40× |
| *CD5* | 422 | 105 (24.9) | 199 (47.2) | 75 (17.8) | 39 (9.3) | 4 (0.9) |
| *CD8A* | 606 | 141 (23.3) | 332 (54.8) | 84 (13.9) | 23 (3.8) | 26 (4.3) |
| *TROP1* | 451 | 240 (53.2) | 185 (41.1) | 21 (5.8) | 0 (0) | 0 (0) |
| *TROP2* | 289 | 155 (53.6) | 99 (34.3) | 24 (8.3) | 8 (2.8) | 3 (1.0) |

The values given are the numbers of clones in each group. The numbers in parentheses are the percentages.

*Ratio of antigen expression on each clone vs. the respective parental cells. <1× means lower expression than the parental transfectant. 1× means no change in surface expression.

Table 2. Surface expression of the transfected genes in clone families by FACS analysis

| Gene | Clone families analyzed | Final level of surface expression after FACS selection* | | | |
|---|---|---|---|---|---|
| | | 1× | 2–4× | 5–10× | 11–40× |
| *CD5* | 10 | 1 | 2 | 5 | 2 |
| *CD8A* | 13 | 0 | 4 | 4 | 5 |
| *TROP1* | 9 | 7 | 2 | 0 | 0 |
| *TROP2* | 6 | 1 | 1 | 2 | 2 |

The values given are the highest increase in surface expression observed in each clone family.
*Ratio of antigen expression of each clone vs. the respective parental cells. 1× means no change in surface expression.

The *TROP1* gene possesses the unusual property of being transfected very efficiently by all the DNA preparations tested, whether the original cellular source expresses the gene or not (10). DNA methylation can affect the efficiency of transfection of a gene (10). Thus, *TROP1* is likely to possess an unusual pattern of methylation, which may also influence its ability to be amplified. To test the hypothesis that genomic DNA methylation affects the frequency of amplification of the *TROP1* gene, we treated the JAR choriocarcinoma cell line with 5-azacytidine, a DNA demethylating drug (10). Efficient demethylation was found in DNA extracted from 5 days to 6 weeks after the treatment as shown by Southern blot analysis and by frequent functional reactivation of methylation-sensitive genes (10). We transfected control JAR DNA and obtained four independent transfectants, none of which was amplified. Sixteen independent Trop-1 transfectants were obtained using demethylated DNA. Most of these showed quite heterogenous expression of Trop-1, unlike most nondemethylated Trop-1 transfectants. None of the three transfectants obtained using DNA extracted at day 5 was amplified. Nine transfectants were obtained with DNA extracted at day 12, and amplification was seen in five of them, reaching up to 40 copies per haploid genome (Fig. 6). With DNA extracted at 6 weeks, four transfectants were obtained. One of them was amplified to 30 copies per haploid genome. Four of the amplified transfectants were selected two more times by sorting the 0.1% brightest cells. In at least two transfectants, a further increase of the copy number of the *TROP1* gene was apparent (Fig. 6, lanes 6–9).

## DISCUSSION

The DNA sequence *per se* appears to be a major determinant in gene amplification (3). In the present article, we show that DNA methylation (24) also affects the frequency of gene amplification.

Table 3. Distribution of the levels of amplification of the transfected genes by Southern blot analysis

| Gene | Clone families analyzed | Final gene copy number after FACS selection* | | | | |
|---|---|---|---|---|---|---|
| | | 1× | 2× | 3–6× | 7–12× | 13–40× |
| *CD5* | 5 | 1 | 1 | 1 | 2 | 0 |
| *CD8A* | 6 | 0 | 1 | 1 | 2 | 2 |
| *TROP1* | 4 | 2 | 2 | 0 | 0 | 0 |
| *TROP2* | 4 | 2 | 0 | 1 | 0 | 1 |

The values given are the number of clone families in each level of amplification.
*Copy number of each transfected gene. Gene copy number was determined by densitometry of Southern autoradiographs, comparing each hybridization signal with that of PBL DNA. 1× means no amplification.

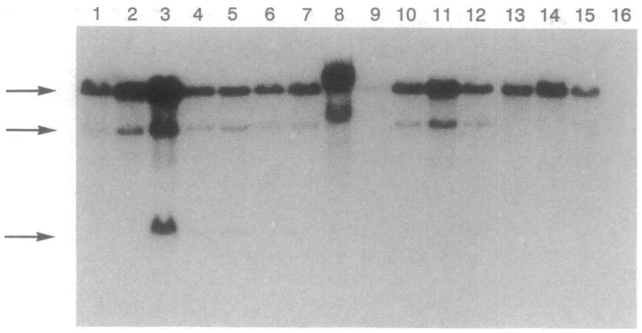


FIG. 2. Southern blot analysis of DNA extracted from CD5 transfectants. Lane 1, JM06*leo*; lane 2, JM06*leo*.6; lane 3, JM06*leo*.6.13; lane 4, JM09*leo*.3; lane 5, JM09*leo*.3.1; lane 6, JM10*leo*; lane 7, JM10*leo*.1; lane 8, JM10*leo*.1.7; lane 9, JM14*leo*; lane 10, JM14*leo*.13; lane 11, JM14*leo*.13.15; lane 12, JM14*leo*.13.20; lane 13, JM20*leo*.6; lane 14, JM20*leo*.6.2; lane 15, human peripheral blood lymphocytes (PBLs); lane 16, unselected transfected L cells. Lanes 1–3, JM06*leo* family; lanes 4 and 5, JM09*leo* family; lanes 6–8, JM10*leo* family; lanes 9–12, JM14*leo* family; lanes 13 and 14, JM20*leo* family. DNA samples were digested with *Eco*RI. Arrows (from the top) indicate 21 kb, 8 kb, and 3 kb.

We compared the amplification ability after transfection of *CD8A* (6, 13), *CD5* (12), *TROP1* (15, 16), and *TROP2* (17, 22). Since sorting for increased surface expression can select for higher copy number of the transfected gene (6), we sorted by cloning the transfectants with the highest gene expression. A clear increase in surface expression was detected in the majority of CD5, CD8α, and Trop-2 transfectant families. No comparable increase was seen for Trop-1. Southern blot analysis indicated frequent gene amplification in the majority of the CD5, CD8α, and Trop-2 transfectant families analyzed.

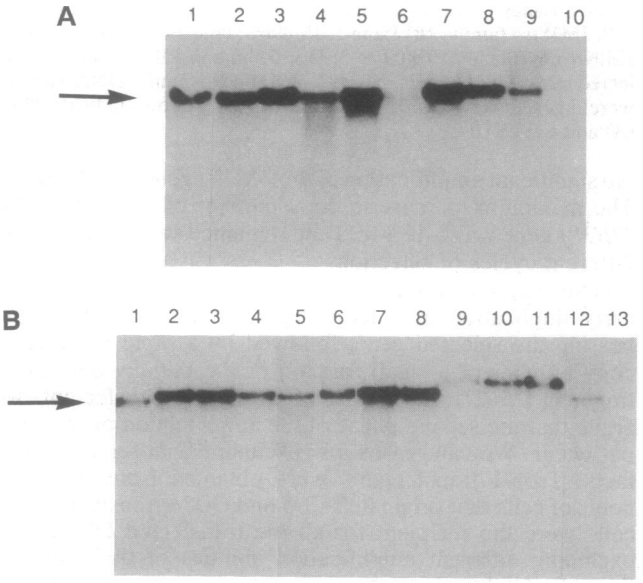


FIG. 3. Southern blot analysis of DNA extracted from genomic CD8α transfectants. (*A*) Lane 1, JM11*let*; lane 2, JM11*let*.11; lane 3, JM11*let*.11.1; lane 4, JM12*let*.6; lane 5, JM12*let*.6.10; lane 6, JM16*let*; lane 7, JM16*let*.1; lane 8, JM16*let*.1.20; lane 9, human PBLs; lane 10, unselected transfected L cells. Lanes 1–3, JM11*let* family; lanes 4 and 5, JM12*let* family; lanes 6–8, JM16*let* family. (*B*) Lane 1, JM14*let*.17.8; lane 2, JM14*let*.17.9; lane 3, JM14*let*.17; lane 4, JM14*let*; lane 5, JM13*let*.4.7; lane 6, JM13*let*.4.2; lane 7, JM13*let*.4; lane 8, JM13*let*; lane 9, JM10*let*.7.4; lane 10, JM10*let*.7.2; lane 11, JM10*let*.7; lane 12, human PBLs; lane 13, unselected transfected L cells. Lanes 1–4, JM14*let* family; lanes 5–8, JM13*let* family; lanes 9–11, JM10*let* family. DNA samples were digested with *Eco*RI. Arrows indicate 9.5 kb.

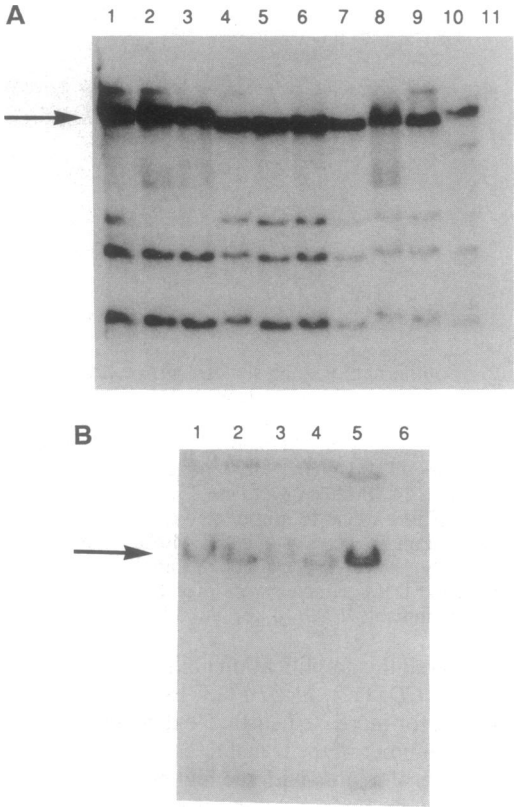


FIG. 4. Southern blot analysis of DNA extracted from genomic Trop-1 transfectants. (*A*) Lane 1, JM14*tro*; lane 2, JM14*tro*.4; lane 3, JM14*tro*.4.17; lane 4, JM17*tro*; lane 5, JM17*tro*.10; lane 6, JM17*tro*.10.1; lane 7, JM18*tro*; lane 8, JM18*tro*.12; lane 9, JM18*tro*.12.2; lane 10, human PBLs; lane 11, unselected transfected L cells. Lanes 1–3, JM14*tro* family; lanes 4–6, JM17*tro* family; lanes 7–9, JM18*tro* family. (*B*) Lane 1, JM09*tro*; lane 2, JM09*tro*.6; lane 3, JM09*tro*.6.7; lane 4, JM09*tro*.3; lane 5, human PBLs; lane 6, unselected transfected L cells. Lanes 1–4, JM09*tro* family. DNA samples were digested with *Bam*HI (*A*) or *Eco*RI (*B*). Arrows indicate 20 kb (*A*) and 6.5 kb (*B*).

No significant amplification of the *TROP1* gene was detected. The maximum increase in copy number of the transfected *TROP1* gene was 2- to 3-fold and remained stable through the different cycles of selection.

Gene amplification consists of at least two phases—i.e., an early duplication of a large segment of the chromosome bearing the selected gene, followed by a rapid increase in copy number of a small fraction of the initially duplicated fragment (25–27). In the case of the Trop-1 transfectants we argue that the second phase of the amplification process did not occur. We can reasonably exclude trivial selection artifacts. Trop-1 transfectants were obtained from the same pools of cells that originated CD5 and CD8$\alpha$ transfectants. L cells were the recipient for all the transfected DNA, thus excluding different amplification abilities of different cell lines (4). All the various transfectants were stained for FACS selection with noncytotoxic purified monoclonal antibodies. Finally, cell survival was not statistically different between Trop-1-expressing cells and the other groups of transfectants, nor was survival significantly affected by different levels of expression of the *TROP1* gene.

*TROP1* is the only gene studied here that is transfected by apparently any source of human DNA (10). DNA methylation can affect the transfection ability of genes (10). Thus, we tested the hypothesis that DNA methylation also prevents amplification of *TROP1*.

We transfected L cells with demethylated DNA and obtained 16 independent Trop-1 transfectants. Southern blot

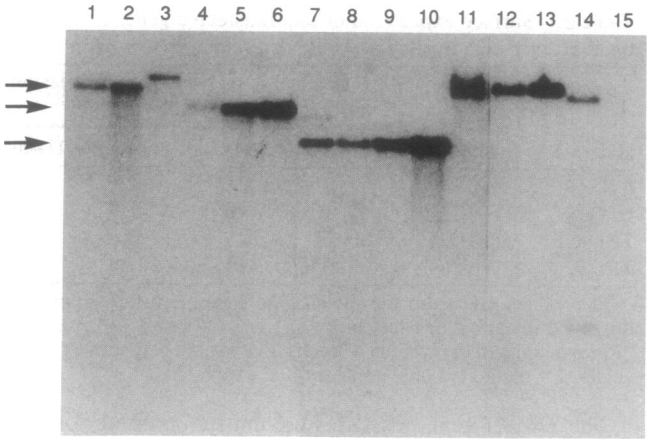


FIG. 5. Southern blot analysis of DNA extracted from genomic Trop-2 transfectants. Lane 1, BEWO07*trt*.8; lane 2, BEWO07*trt*.8.6; lane 3, BEWO08*trt*; lane 4, BEWO08*trt*.16; lane 5, BEWO08*trt*.16.21; lane 6, BEWO08*trt*.16.21.1; lane 7, BEWO09*trt*; lane 8, BEWO09*trt*.14; lane 9, BEWO09*trt*.14.1; lane 10, BEWO09*trt*.14.4; lane 11, BEWO11*trt*; lane 12, BEWO11*trt*.6; lane 13, BEWO11*trt*.6.1; lane 14, human PBLs; lane 15, unselected transfected L cells. Lanes 1 and 2, BEWO07*trt* family; lanes 3–6, BEWO08*trt* family; lanes 7–10, BEWO09*trt* family; lanes 11–13, BEWO11*trt* family. DNA samples were digested with *Eco*RI. Arrows (from the top) indicate 20 kb, 11 kb, and 7.5 kb.

analysis proved that demethylated *TROP1* genes are amplified efficiently. The highest efficiency of amplification was obtained with DNA extracted from JAR cells 12 days after the treatment with 5-azacytidine. The same DNA preparation also possesses the highest efficiency of transfection of methylation-sensitive genes (10). The amplification of the *TROP1* gene is also progressive, similar to what is commonly observed in CD5, CD8$\alpha$, and Trop-2 amplificants.

DNA methylation may affect amplification in multiple ways (44). First, it may interact with DNA replication (1, 5, 7, 25, 28). DNA methyltransferases interact with the DNA replication machinery (24, 29). DNA methylation can affect the interaction of transcription factors with DNA (24, 30–33), and transcriptional elements can be components of origins of DNA replication (34). Moreover, DNA methylation could affect DNA recombination. Indeed, cytosine methylation modulates the interaction of repair enzymes with DNA (35), and altered DNA methylation has been found in regions of chromosomal rearrangement (36). Further, DNA methylation alters the frequency of recombination in immunoglobulin genes after DNA replication (37) and affects the pattern of integration of retroviruses in different sites of the genome (38) and may cause different amplification in different sites in the genome (40). DNA methylation could influence either replication- or recombination-mediated amplification by affecting binding of proteins to methylcytosines (30, 31) or by affecting chromatin conformation (39).

In our amplifications we have some evidence for involvement of a recombination mechanism. In all clone families where some clones lost copies of the transfected gene, we also observed clones that were amplified. This suggests the idea of a sister chromatid exchange-like mechanism (25).

In tumor cells, alterations of both DNA methylation (41) and amplification of oncogenes (2, 5, 7, 8) or of drug resistance genes (2, 5, 27) are frequent, unlike the case in normal cells (42, 43). Similarly, alterations in DNA methylation and gene amplification (24, 41–43) are concurrent in cell lines in culture. If DNA methylation is a determinant of gene amplification *in vivo*, it may be possible to influence gene amplification in tumor cells through a modulation of DNA methylation.

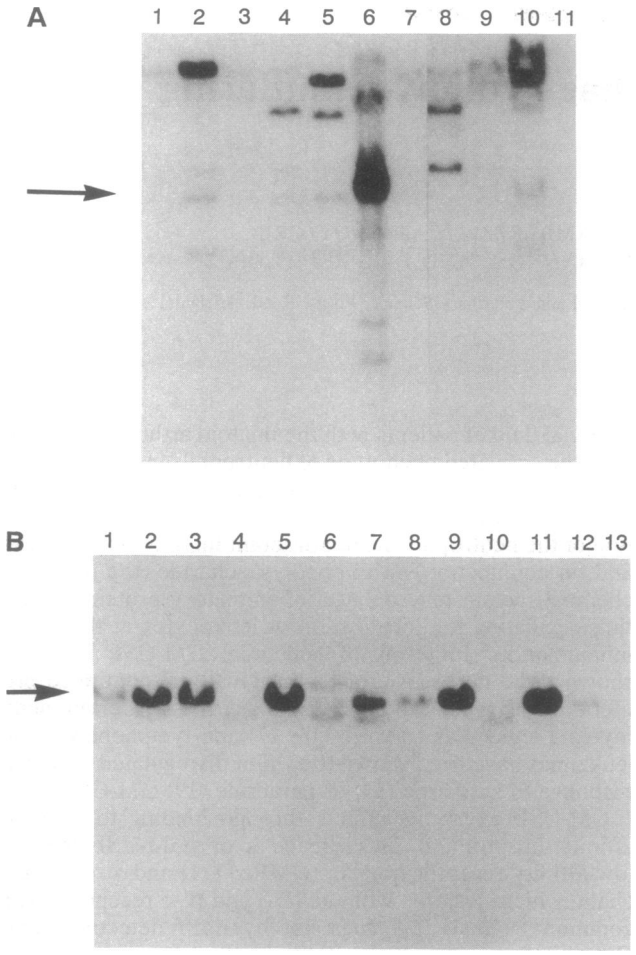


FIG. 6. Southern blot analysis of DNA extracted from genomic Trop-1 transfectants obtained using demethylated JAR DNA. (*A*) Lane 1, JAR03*tro*; lane 2, JARaza5D10*tro*; lane 3, JARaza5D03*tro*; lane 4, JARaza5D09*tro*; lane 5, JARaza12D26*tro*; lane 6, JARaza12D23*tro*; lane 7, JARaza6W06*tro*; lane 8, JARaza6W06*tro*; lane 9, JARaza6W26*tro*; lane 10, human PBLs; lane 11, unselected transfected L cells. (*B*) Lane 1, JARaza12D01*tro*; lane 2, JARaza12D03*tro*; lane 3, JARaza12D03*tro*/2; lane 4, JARaza12D04*tro*; lane 5, JARaza12D05*tro*/2; lane 6, JARaza12D06*tro*; lane 7, JARaza12D06*tro*/2; lane 8, JARaza12D08*tro*; lane 9, JARaza12D08*tro*/2; lane 10, JARaza12D10*tro*; lane 11, JARaza6W03*tro*; lane 12, human PBLs; lane 13, unselected transfected L cells. DNA samples were digested with *Bam*HI (*A*) or *Eco*RI (*B*). Arrows indicate 4.5 kb (*A*) and 5.5 kb (*B*). Note that the exposure time in *A* was 7 days and that in *B* was 5 hr.

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