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Transfer and expression of herpes simplex virus thymidine kinase and human globin genes in mammalian cells studied by spot hybridization assays

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A spot hybridization assay has been developed for rapid estimations of specific DNA sequences in whole mammalian cells or DNA. This method is applicable to gene-transfer studies in determining the copy number(s) of foreign DNA(s) introduced into mammalian cells.

A similar assay has been developed for determining amounts of specific RNA sequences in mammalian cells. It is also shown that this method is applicable to gene-transfer studies and in particular to determining the expression of foreign genes introduced into cells.

The transformation of mammalian cells with purified DNA fragments of viral or cellular origin has been demonstrated by a number of investigators (1-4). The detection of donor DNA sequences in transformant cell lines usually involves either liquid hybridization or Southern blots. Both of these procedures require prior isolation of DNA from transformed cells and are relatively laborious.

Moreover, studies on the expression of transferred genes require elaborate methods, i.e. Northern blots, S1 mapping, or liquid hybridization for the detection of transcripts, particularly if their level of expression is very low (5-7).

This communication describes a simple quantitative method for determining donor DNA sequences in transformed cell lines using a small number of cells, and a similar method for determining RNA transcripts in total cellular RNA from a gene for which a probe is available. These methods can be used as a fast screening test for transformants, especially in experiments where the transfer of nonselectable markers is attempted, i.e., in cotransformation (8,9) or where transfer of a gene covalently linked to a selectable marker is attempted (5,7), thus avoiding the need for growing large cultures.

Similar techniques have been described recently by others (10-12), and preliminary results have been presented by us elsewhere (13).

In this paper some parameters of the method are defined.

Materials and Methods

Cells and plasmids

Plasmid pTK-1 obtained from Dr. N. M. Wilkie (14) was constructed by inserting a 3.5-kilobase-pair BamHI fragment of herpes simplex virus type 1 containing the thymidine kinase gene into the

BamHI site of plasmid pAT-153 (15). Recombinant plasmid pTKHεG-1 carrying the human embryonic epsilon-globin gene was constructed by inserting a HindIII 8.0-kb human DNA fragment of the recombinant phage λ 788 (16) into the single HindIII site of pTK-1. The same 8.0-kb human DNA fragment was inserted into the HindIII site of plasmid pAT-153 to obtain pHεG-1. Recombinant plasmid pTKHβG-1 carries a 5.0-kb human DNA of HβG2 (17) coding for the beta-globin gene covalently linked to the thymidine kinase gene of HSV-1. Plasmid pHαG-7 was constructed by inserting a 3.8-kb PvuII fragment of λ HαG2 DNA containing the α1 human globin gene (18) into the single PvuII site of pBR-322. Plasmid pHβG-RP carries a 0.8-kb fragment of recombinant phage λ HβG2 (17) containing the EcoRI-PstI fragment of the 3' region of the human beta-globin gene and extragenic region inserted into the EcoRI-PstI sites of pAT-153. Plasmid pHεG-BR carries a 1.3-kb fragment of recombinant phage λ 788 containing the BamHI-EcoRI fragment of the 3' region of the human epsilon-globin gene and extragenic region inserted into the BamHI-EcoRI sites of pAT-153.

DNA was labelled by nick-translation (19) using ^{32}P-labelled nucleotide triphosphates (Amersham).

Thymidine-kinase-deficient mouse (LMTK$^-$) and hamster (BHKTK$^-$) cells were obtained from Dr. N. M. Wilkie, and Friend (F4-12B2TK$^-$) cells from Dr. W. Ostertag. Cells were grown in monolayers in supplemented Ham's SF12 medium (Flow Laboratories) containing 15% fetal calf serum (FCS). Cell lines F501, F502, and F503 were obtained after transformation of F4-12B2K$^-$ Friend cells with the recombinant plasmid pTKHβG-1 as described below.

DNA-mediated gene transfer

We have used the calcium phosphate technique (4,20) with the following modification: our selective medium was SF12 containing 30% FCS, 0.9% methocel (Dow Chemicals), 100 μM hypoxanthine, 0.8 μM aminopterin, and 15 μM thymidine (HAT). Colonies were picked 7-10 d later with a Pasteur pipette and grown in SF12 medium containing 15% FCS and HAT.

Procedure for spot hybridization assay

Two different protocols were used with total cells. In the first, cells were resuspended in 0.5 N NaOH, 1.5 M NaCl, usually at a concentration of 1×10^7 cells/ml. After mixing for a few seconds, a clear lysate was obtained. The nitrocellulose filter (Sartorius or Millipore) was placed on a sintered glass filter, and 5 μl of 0.5 N NaOH, 1.5 M NaCl was applied with suction to the area to which the lysate was to be applied. The samples (usually 5-200 μl) were then applied to the nitrocellulose filter with suction, followed by one drop of 1.0 M Tris-HCl (pH 7.4), 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) per spot. The vacuum was released and the filter placed on Whatman 3 MM paper soaked in 1 M Tris-HCl (pH 7.4), 2 × SSC for 1 min. The filter was transferred to a dry Whatman filter paper and this was repeated. Finally the filter was baked at 80°C for 2 h.

Alternatively the cells were washed with phosphate-buffered saline (PBS) and resuspended at 10^7-10^8 cells/ml of PBS. Replicate 5-µl samples were applied to 1-cm^2 squares marked on a dry nitrocellulose filter. The filter was soaked consecutively in 0.5 M NaOH for 10 min, and twice in 1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4, for 5 min each. The filter was then dried in air for 30 min, washed in $CHCl_3$, and dried in air again. After a final rinse in 3 × SSC, the filter was dried in air and baked at 80°C for 2 h. Filter hybridization was performed as described.

For DNA spot hybridization assays, DNA was dissolved in 1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, at a concentration of 1 mg/ml, and 5-µl aliquots were applied to 1-cm^2 squares on a nitrocellulose filter pretreated with 20 × SSC. The filter was then alkali-treated, neutralized, dried, washed, baked, and hybridized as described elsewhere (21). For RNA spot hybridization assays RNA was dissolved in H_2O at a concentration of up to 4 mg/ml, and 5-µl aliquots were applied to 1-cm^2 squares on a nitrocellulose filter pretreated with 20 × SSC. The filter was then baked for 2-3 h in an 80° oven, prehybridized, and hybridized as described elsewhere (21).

Digestions of DNA and RNA

To determine whether hybridization was due to DNA or RNA, the filter was treated as follows: for DNA digestion the filter was incubated in DNase I at 30 µg/ml in 10 mM $MgCl_2$, 200 mM NaCl, 5 mM phosphate buffer, pH 6.0, for 4 h at 37°C. It was then washed in 30 mM EDTA, 3 × SSC and dried. For RNA digestion the filter was incubated for 2 h at 37°C in RNase (heated to 90°C for 10 min) at 100 µg/ml in 1 × SSC. The filter was then washed with 3 × SSC, dried, and hybridized.

Results and Discussion

Capacity of nitrocellulose filters for binding lysed cells

Mouse LMTK$^+$ cells transformed with pTK-1 DNA were lysed in 0.5 N NaOH, 1.5 M NaCl at a concentration of 1 × 10^7 cells/ml, and 10, 50, 100, and 200 µl were spotted onto the nitrocellulose filter as described in 'Materials and Methods'. The DNA bound to the filter was hybridized with nick-translated ^{32}P-pTK-1 DNA and the results are shown in Fig. 1. As seen in the autoradiograph of Fig. 1a, no cross-hybridization was observed with recipient mouse LMTK$^-$ cells at all the cell concentrations used. On the other hand, with all three transformant cell lines, the size and intensity of the spots increased with an increasing number of cells spotted. T4 cells gave more intense spots than T3 or T16 at the same cell concentrations. As shown later by Southern blot analysis, this is due to more pTK-1 molecules/cell. Each of the 16 squares of the filter was cut out and the ^{32}P bound to it was counted by liquid scintillation. Four squares not spotted with cells were counted to measure background radioactivity. Thus the radioactivity in each spot could be quantitated, and the results are shown in Fig. 1b. As seen in the figure, all cell concentrations tested (1-20 × 10^5 cells/spot) gave a linear response. A simpler and inexpensive way to estimate the radioactivity in the spots is shown in Fig. 1c, where a scan of the autoradiograph of spots

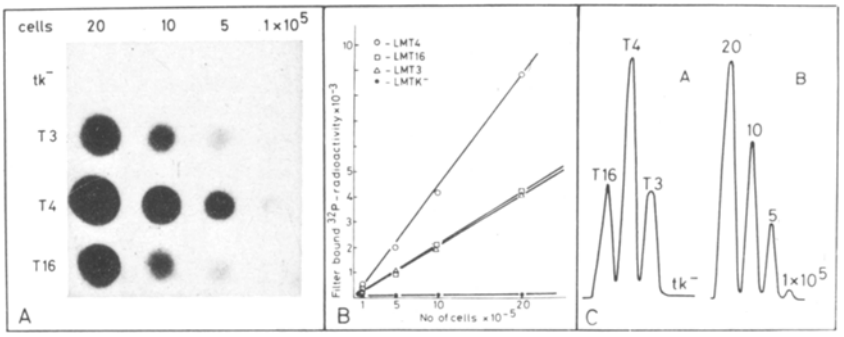


Fig. 1. Spot hybridization assay for whole cells. Cells were lysed in 0.5 N NaOH, 1.5 M NaCl and spotted on the nitrocellulose filter under suction according to the first protocol as described in 'Materials and Methods'. $LMTK^+$ transformed cell lines T3, T4, and T16 were obtained after transformation of $LMTK^+$ cells with pTK-1 DNA as described in 'Materials and Methods'. The autoradiograph and the arrangement of cells are shown in (a). The probe was 1×10^6 c.p.m. of nick-translated ^{32}P-pTK-1 (sp. act. 1×10^7/μg DNA). The filter was exposed to X-Omat X-ray film (Kodak) for 4 h at -70°C using a cronex intensifying screen (Dupont). (b) Quantitation of the amount of ^{32}P-pTK-1 radioactivity bound to the filter in the spot hybridization assay shown in (a). After autoradiography, each spot was cut out and ^{32}P was estimated by liquid scintillation counting. (c) Quantitation of spot hybridization assay by scanning of the autoradiograph shown in (a); the scans of the spots corresponding to concentrations of 20×10^5 cells per spot are shown in (A), whereas in (B) are shown the scans of T3 transformant cells at the indicated concentrations per spot.

at a concentration of 20×10^5 cells as shown in (A) and a scan of spots derived from various concentrations of T3 transformed cells in (B) are consistent with the amounts of ^{32}P radioactivity estimated by scintillation counting. These results indicate a linear correlation between the number of cells lysed and the amount of probe DNA bound to the nitrocellulose filter.

To examine whether the hybridization was detecting pTK-1 DNA or RNA transcripts of it, untreated and transformed erythroleukemic Friend cells, transformed with a recombinant carrying a human beta-globin gene (pTKHβG-1), were lysed and spotted in triplicate together with markers of human globin DNA on a background of recipient cells. The first filter was left untreated, the second was treated with DNase, and the third with RNase (Fig. 2). Filters were

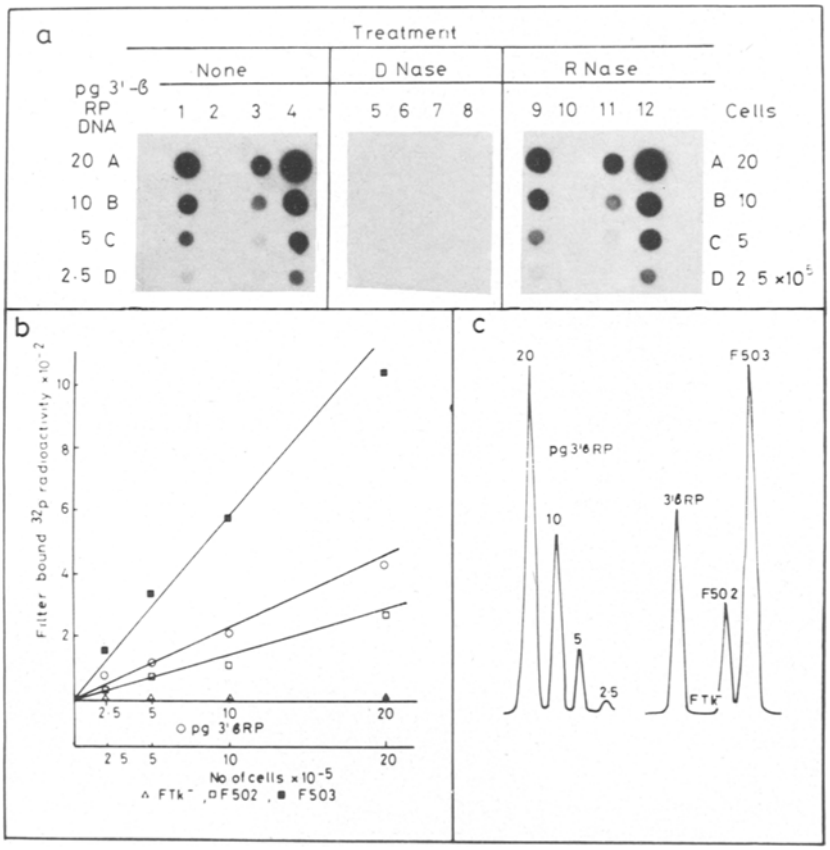


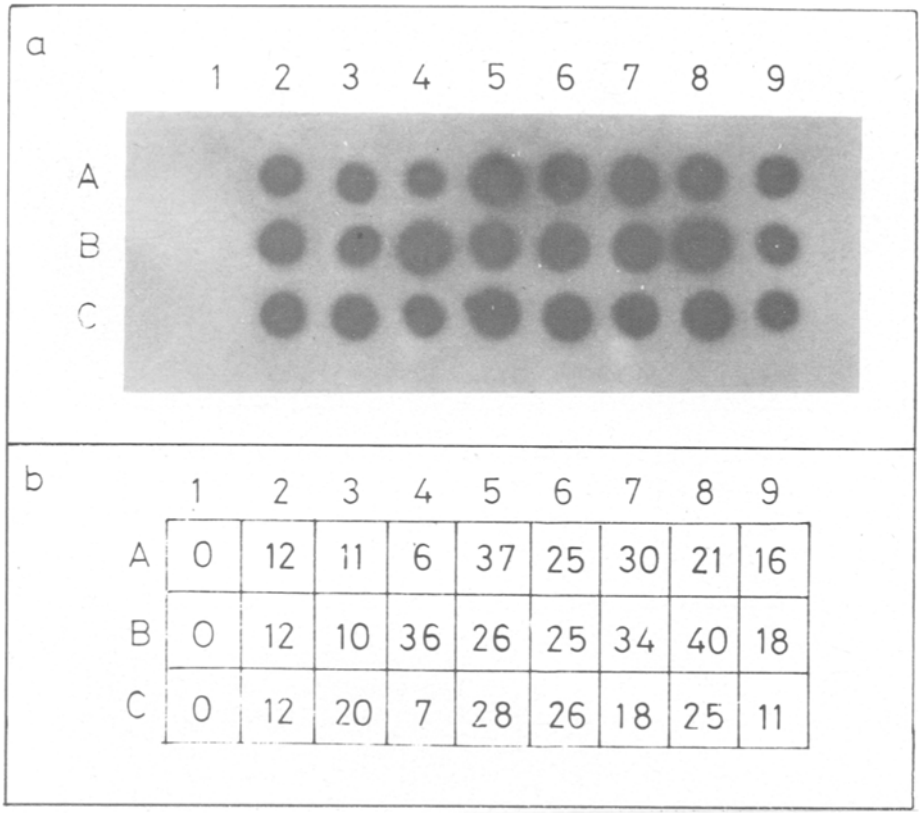
Fig. 2. Effects of DNase and RNase treatment of spot hybridization of transformed Friend cells. Cells were spotted onto nitrocellulose as described in protocol 2 in 'Materials and Methods'. The autoradiograph and the arrangment of cells, marker DNA, and treatments are shown in (a). Spots 1, 2, 3, and 4 (A, B, C, D) represent untreated filter, whereas 5, 6, 7, and 8 (A, B, C, D) and 9, 10, 11, and 12 (A, B, C, D) represent replicas of the first filter treated with DNase and RNase respectively. (b) Quantitation of the amount of ^{32}P-3'βRP DNA bound to the filter in the spot hybridization assay shown in (a). After autoradiography, each spot was cut out and ^{32}P was counted by liquid scintillation. (c) Quantitation of DNA spot hybridization assay by scanning of the autoradiograph shown in (a). 1 (A-D) represent 20, 10, 5, and 2.5 pg of 3'-βRP DNA fragment on a background of 20, 10, 5, and 2.5 × 10^5 F4-12B2TK$^-$ cells respectively; 2 (A-D), F4-12B2TK$^-$ cells; and 3 and 4 (A-D), F502 and F503 transformed cells as indicated.

then hybridized with a ^{32}P-labelled nick-translated human beta-globin DNA fragment containing the 3' end of the gene and the extragenic sequences (3'-βRP 0.8-kb DNA fragment was obtained from plasmid pHβG-RP after sequential digestion with EcoRI and PstI and isolation through a 5-20% sucrose gradient). As shown in Fig. 2, RNase digestion did not reduce the extent of hybridization whereas DNase digestion totally eliminated hybridization.

Identification of nonselectable cotransferred DNA sequences in transformant cell lines

We have isolated several LMTK$^+$, BHKTK$^+$, and F4-12B2TK$^+$ cell lines transformed with the chimeric plasmid pTKHεG-1 DNA. These were selected in medium containing HAT for transfer of the thymidine kinase gene of herpes simplex virus type 1. To examine whether the transformant clones also contained human ε-globin sequences, a spot hybridization assay was carried out using 1×10^5 cells of each transformant/spot and hybridized with a nick-translated ^{32}P-DNA fragment containing the 3' end of the gene and the extragenic sequences (3'-εBR DNA) which provide a specific probe for the human epsilon-globin gene. The 3'-εBR 1.3-kb DNA fragment was obtained from plasmid pHεG-BR after sequential digestion with BamHI and EcoRI and isolation through a 5-20% sucrose gradient. The autoradiograph is shown in Fig. 3a and the quantitation in Fig. 3b. It can be seen that all LM (A3-A9), BHK (B3-B9), and F4-12B2 (C3-C9) TK$^+$ transformed cell lines carry multiple copies of the 3'-εBR DNA sequences.

It has been reported that competent mammalian cells transformed with a selectable biochemical marker can take up and become stably transformed with procaryotic and eucaryotic genes for which no selective criteria exist (8,9). We have performed similar cotransformations of BHKTK$^-$ cells with pTK-1 and recombinants containing either human epsilon- or alpha-globin genes in pAT-153 and pBR, respectively. Spot hybridization assays were used to determine the presence of nonselectable cotransformed human epsilon- and alpha-globin sequences into BHKTK$^+$ cells. As shown in Fig. 4a, 4 out of 10 BHKTK$^+$ transformed clones exposed to epsilon recombinant DNA carry human epsilon-globin sequences, and as shown in Fig. 4b, 6 out of 10 BHKTK$^+$ transformed clones exposed to alpha recombinant DNA carry human alpha-globin sequences. Our results on cotransformation of BHKTK$^-$ cells are similar to those obtained by others (8,9) on cotransformation of mouse LMTK$^-$ cells. That is, although a 1000-fold excess of the nonselectable DNA is added during cotransformation, only 40-60% of the transformed cell lines carry nonselectable DNA molecules. Although the mechanisms of cotransformation are not known (22), the fact that mouse and hamster recipient cell lines behave similarly suggests that it is a more general phenomenon and not restricted to a particular cell line. On the other hand, spot assay hybridization is shown here to be of practical importance for a fast and inexpensive screening of cotransferred sequences in the recipient cells.



	1	2	3	4	5	6	7	8	9
A	0	12	11	6	37	25	30	21	16
B	0	12	10	36	26	25	34	40	18
C	0	12	20	7	28	26	18	25	11

Fig. 3. Determination by the spot hybridization assay of human epsilon-globin DNA sequences in LMTK$^+$, BHKTK$^+$, and F4-12B2TK$^+$ cell lines transformed with pTKHεG-1 DNA is shown in (a). 5 µl containing 1×10^5 cells was placed on each spot. Spots A1, B1, and C1 represent recipient LMTK$^-$, BHKTK$^-$, and F4-12B2TK$^-$ cell lines, respectively. A2, B2, and C2 represent 20 pg of pTKHεG-1 DNA on a background of 1×10^5 LMTK$^-$, BHKTK$^-$, or F4-12B2TK$^-$ cells. Spots A3-A9, B3-B9, and C3-C9 represent independently isolated LMTK$^+$F, BHKTK$^+$, and F4-12B2TK$^+$ transformant cell lines, respectively. Each filter was hybridized with 3×10^7 c.p.m. of nick-translated ^{32}P-labelled 3'-εBR DNA (sp. act. 1×10^8 c.p.m./µg DNA). After autoradiography, each spot was cut out and ^{32}P was counted by liquid scintillation. The estimated number of 3'-εBR 1.3-kb DNA copies/cell is shown in (b).

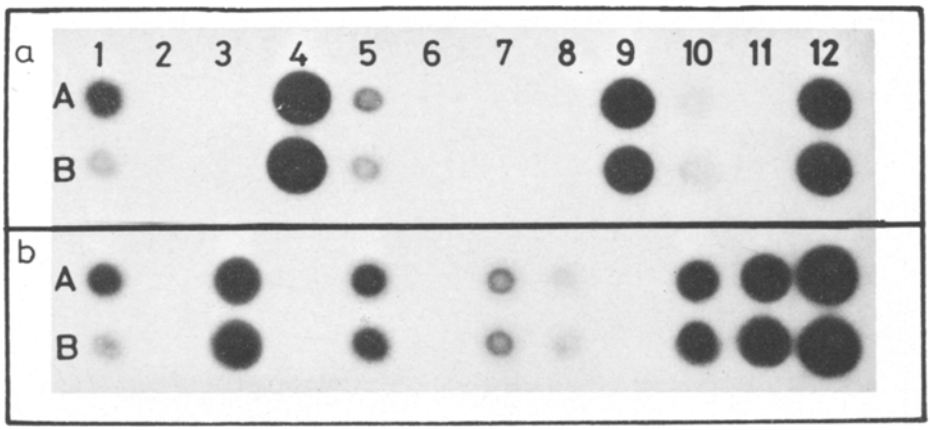


Fig. 4. Spot hybridization assay to determine the presence of cotransformed human epsilon- and alpha-globin sequences in BHKTK$^+$ cells transformed with pTK-1 DNA. (a) 1 (A,B) represent 100 and 50 pg of pHεG-1 DNA on a background of 2 × 10^5 BHKTK$^-$ cells, and 2 (A,B), 2 × 10^5 BHKTK$^-$ cells. 3-12 (A,B) are duplicate samples of 2 × 10^5 BHKTK$^+$ cells cotransformed with 20 ng of pTK-1 and 20 µg of pHεG-1 DNA. (b) 1 (A,B) represent 100 and 50 pg of pHαG-7 DNA on a background of 2 × 10^5 BHKTK$^-$ cells, and 2 (A,B), 2 × 10^5 BHKTK$^-$ cells alone. 3-12 (A,B) are duplicate samples of 2 × 10^5 BHKTK$^+$ cells of individual clones cotransformed with 20 ng of pTK-1 and 20 µg of pHαG-7 DNA. Nick-translated ^{32}P-HεG (8.0-kb HindIII fragment of pHεG-1) or ^{32}P-HαG (3.8-kb PvuII fragment of pHαG-7) DNA were used as probes in (a) and (b), respectively.

RNA spot hybridization assay

RNA preparations isolated from untransformed and transformed Friend erythroleukemic cells as well as purified RNA from fetal exchange blood containing 20% β-globin mRNA were spotted in duplicate at the indicated dilutions onto nitrocellulose filters pretreated with 20 × SSC (Fig. 5). The first filter was left untreated whereas the second was treated with RNase. Filters were then hybridized with a ^{32}P-labelled nick-translated DNA fragment containing the 3' end of the β-globin gene. As shown in Fig. 5, RNase eliminated hybridization in the second filter. In the untreated filter, no cross-hybridization was observed with RNA from recipient cells and a linear dose-response relationship was observed with both β-globin mRNA and RNA from transformed cells.

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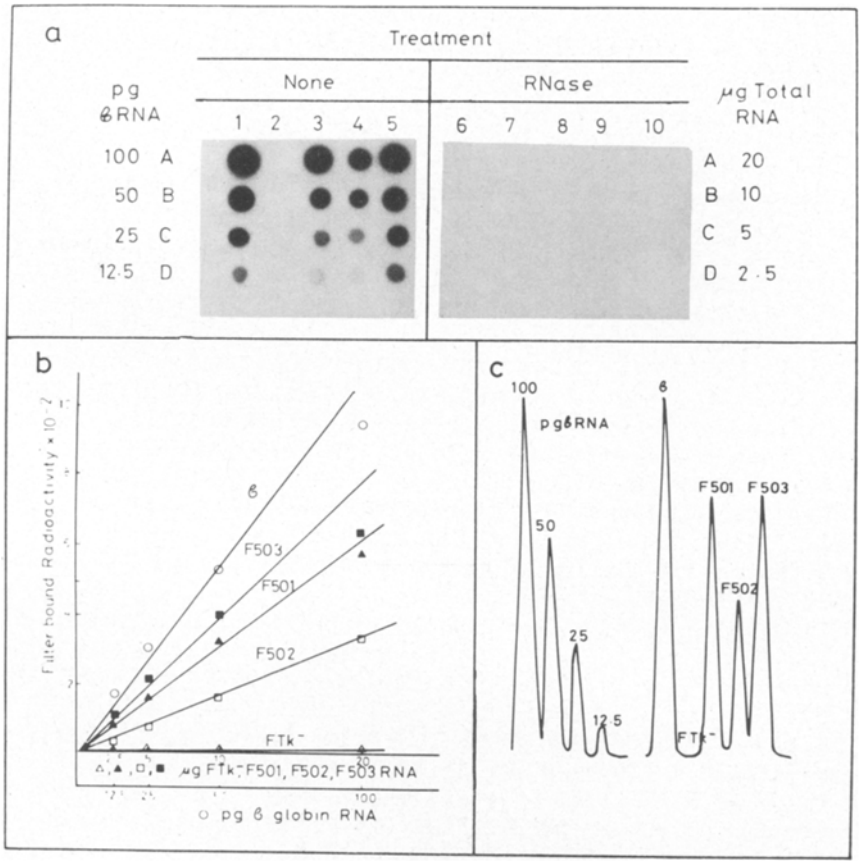

Fig. 5. Effect of RNase treatment on spot hybridization of RNA from transformed Friend cells. The RNA, prepared by a standard phenol extraction method, was spotted on a nitrocellulose filter as described in 'Materials and Methods'. The autoradiograph and the arrangment of RNAs are shown in (a). (b) Quantitation of the amount of ^{32}P-3'βRP DNA bound to the filter in the spot hybridization assay shown in (a). After autoradiography, each spot was cut out and ^{32}P was counted by liquid scintillation. (c) Quantitation of the RNA spot hybridization assay by scanning of the autoradiograph shown in (a) row A. 1 (A-D) represent 100, 50, 25, and 12.5 pg of human β-globin mRNA on a background of 20, 10, 5, and 2.5 μg of total RNA from F4-12B2TK$^-$ cells; 2 (A-D) represent the same amounts of total RNA from F4-12B2TK$^-$ cells alone; and 3 (A-D), 4 (A-D), and 5 (A-D), RNA from F501, F502, and F503 transformed cells as indicated.

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