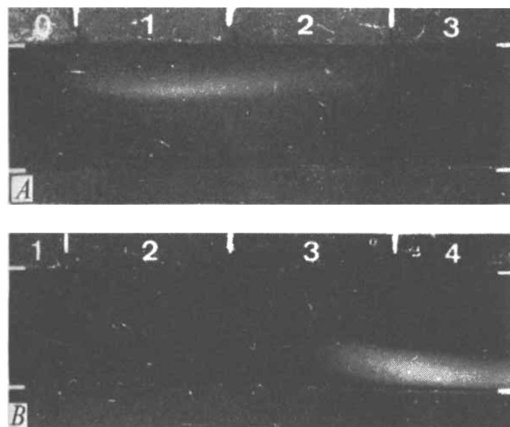

Fig. 1 The limits between the 6 gel layers in each of the two cells (double diffusion) are indicated by white dashes. The lower layer was that of an anti-idiotypic serum (different in the two cells). The intermediate layers contained pure agar. The upper layers 1–4 in the two cells were those of anti-SAE sera of successive bleedings of two rabbits; layer O was that of serum collected before anti-SAE immunization. In the *A* cell, an idiotype disappeared progressively from bleedings 1–3 (still strongly anti-SAE). In the other cell (*B*), an idiotype appeared between bleedings 3 (already strongly anti-SAE) and 4.

gave no visible reaction with the six anti-idiotypic sera used; among the reactions of the other twelve anti-SAE sera with these six anti-idiotypic sera, twenty-three were positive in liquid medium, and nine of them were visible in gels.

Each heterologous reaction, as well as each homologous reaction, does not occur if the anti-SAE serum is replaced by the normal serum of the same rabbit or absorbed with the SAE somatic antigen. An anti-idiotypic serum absorbed with the anti-SAE homologous serum does not give heterologous reactions, but the converse is not true; as in cross-reactions, its absorption with a heterologous anti-SAE serum may change the appearance of one or several precipitation zones in its reaction with the homologous anti-SAE serum without making them disappear.

In spite of the heterologous reactions, therefore, each idiotypic pattern (made of an unknown number of determinants) of anti-SAE antibodies seems to be peculiar to a given individual, although a given idiotypic determinant (or related determinants) may occur in anti-SAE antibodies of different rabbits. Only partial similarity of structure of variable regions of the immunoglobulin polypeptide chains in the homologous and the heterologous idiotype seems to be expressed by heterologous reactions as though there were only partial similarities of the genes coding for them.

Applications to several aspects of idiotypy have been made possible by the reactions of each anti-idiotypic serum with anti-SAE sera other than the immunizing one, the number of these sera being very large in theory.

Using homologous reactions, idiotypic specificities of anti-*S. typhi* antibodies of one given individual are not detected in the anti-*S. typhi* antibodies of either of its parents[6,7]. Using heterologous reactions, in three different litters, several anti-idiotypic sera which precipitated the anti-SAE serum of one given rabbit precipitated the anti-SAE serum of neither parents. Moreover, the heterologous reactions of one anti-idiotypic serum do not seem to be less frequent with the anti-SAE sera of the rabbits of a litter the parents of which gave anti-SAE sera not precipitated by the considered anti-idiotypic serum (compared with anti-SAE sera of randomly chosen rabbits of unknown parents).

Comparing heterologous reactions of anti-idiotypic sera confirms that several rabbits similarly and successfully immunized do not always form antibodies against the same idiotypic determinants. New idiotypes may appear in the course of anti-*S. typhi* immunization[6,7] and indirect signs of disappearance of idiotype(s) have been observed[6,7,14]. Two examples of disappearance of idiotypes, one of which is shown in Fig. 1*A*, and one instance of appearance of an idiotype (Fig. 1*B*) have been observed with the help of heterologous reactions.

JACQUES OUDIN
GUY BORDENAVE

Service d'Immunochimie Analytique,
Institut Pasteur,
Paris

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Suppression of Immunoglobulin Synthesis by Cellular Hybridization

WHEN two cells which show differentiated characteristics are fused, the hybrids generally do not show these characteristics. Ephrussi *et al.*[1] have interpreted such *trans* dominant loss to show that the cells contain a diffusible repressor, and this idea is supported by the finding that some mouse/human hybrids can spontaneously redifferentiate when the C10 human chromosome is lost[2]. We now report another example of redifferentiation in hybrid cells; when 1T mouse fibroblasts are fused with MPC 11 mouse myeloma cells which produce immunoglobulin, the hybrids neither produce nor excrete detectable amounts of immunoglobulin.

MPC 11 mouse cells grow in suspension and do not attach to plastic or glass[3]; about 30% of the protein which these cells synthesize is immunoglobulin. At the time of fusion, more than 99% of the cells were producing heavy (H) and all were producing light (L) chains, as judged by cloning experiments[4]. The mouse fibroblasts derive from a 3T3 line which lack thymidine kinase[5].

Cells were fused by incubating 7×10^6 MPC 11 cells with 1.5×10^6 1T cells in 2 ml. Hanks balanced salt solution for 0.5 h at 4° C and then for another 0.5 h in a shaking water bath at 37° C. The cells were seeded in four 60 mm Petri dishes in normal growth medium and 24 h later were fed with a medium (GHAT) containing aminopterin to select against the 1T fibroblast line[6]. The parent MPC 11 cells were removed by repeated washing with medium. After 18 days, six hybrid colonies which grew attached to the glass and had survived in the selective medium were isolated. Each colony was presumably the result of a separate fusion. We made a preliminary survey of immunoglobulin production less than 2 months after fusion and completed all studies within 5 months of fusion.

The hybrid nature of these cells was shown by their ability to grow in selective medium, their fibroblast-like morphology, their chromosome numbers, uptake of ^{14}C-thymidine, and the presence of H-2 histocompatibility antigens from both parents. The mean number of chromosomes in both MPC 11 and 1T

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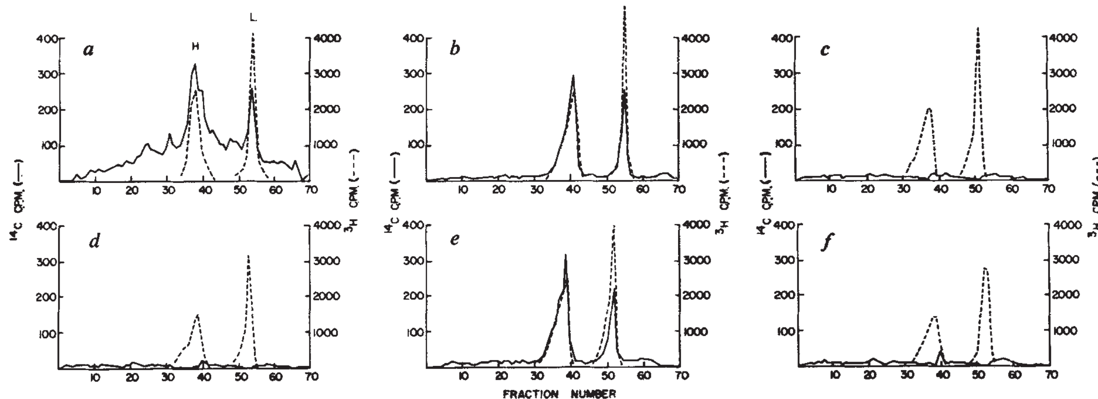

Fig. 1 Cytoplasmic protein of MPC 11 hybrid colony 4 and reconstruction experiment, 6 : 1 ratio. MPC 11 cells were washed twice in Eagle's medium containing 1/40 the normal concentrations of valine, threonine and leucine and 5×10^6 cells were incubated for 30 min at 37° C in 1 ml. of the same medium containing 5 μCi each of ^{14}C-valine, ^{14}C-threonine and ^{14}C-leucine[3]. To prepare cytoplasm[14] the cells were lysed with 0.5% 'Nonidet P-40' (NP-40) and nuclei and microsomes removed by centrifugation at 100,000*g* for 30 min. A nearly confluent Blake bottle of colony 4 (about 10^7 cells) was labelled in 8 ml. of medium containing 20 μCi of each of the same ^{14}C-labelled amino-acids for 30 min, at 37° C, on a rocker platform. Cytoplasm was prepared as above, except that the lysis mixture also contained 0.5% deoxycholate. The amount of TCA precipitable radioactivity was determined. MPC 11 cytoplasm was divided into 6,000 c.p.m. aliquots and colony 4 cytoplasm into 36,000 c.p.m. aliquots. One aliquot of MPC 11 was mixed with ^{3}H-labelled MPC 11 marker, made to 2% with SDS and 0.15 M with 2-ME and after treatment for 1 min at 100° C and 30 min at 37° C was dialysed for 16 h against a solution containing 0.1% SDS, 0.01 M neutral phosphate buffer and 0.015 M 2-mercaptoethanol. The sample was then applied to a 20 cm 5% polyacrylamide gel containing 0.1% SDS[12,13] and electrophoresed for 16 h at 6 mA per gel tube (*a*). Immunologic precipitation was carried out by adding 10 μl. rabbit antiserum at 4° C for 30 min, followed by 0.25 ml. of sheep anti-rabbit gamma globulin for 16 h. Rabbit antiserum prepared against purified MPC 11 myeloma protein was used for specific precipitation and rabbit antiserum prepared against purified penton subunit of adenovirus-2 (from Dr Marshall Horwitz) for nonspecific precipitation. Aliquots of MPC 11 and colony 4 cytoplasm were mixed, marker added and the mixture then immunologically precipitated (*e* and *f*). Aliquots of colony 4 alone were also precipitated (*c* and *d*). The precipitates were washed twice with phosphate buffered saline (0.5 ml.), resuspended in 2% SDS + 0.01 M neutral phosphate and solubilized by heating at 100° C for 1 min. Additional dialysed marker was added to the nonspecifically precipitated samples. Each sample was adjusted to a final concentration of 0.15 M 2-mercaptoethanol, incubated at 37° C for 30 min and electrophoresed as described. Gels were divided into 70 fractions and counted for ^{3}H and ^{14}C label in a Beckman scintillation counter for 10 min. Correction was made for channel overflow and background where significant. *a*, MPC 11 cytoplasm, unprecipitated; *b*, MPC 11 cytoplasm, specifically precipitated; *c*, colony 4 cytoplasm alone, specifically precipitated; *d*, colony 4 cytoplasm alone, nonspecifically precipitated; *e*, MPC 11 plus six-fold excess of colony 4 cytoplasm, specifically precipitated; *f*, MPC 11 plus six-fold excess of colony 4 cytoplasm, nonspecifically precipitated.

was 65. Two months after fusion the number of chromosomes in the hybrids indicated that colonies 1, 2 and 4 were formed by 1 : 1 fusion and colonies 3 and 6 presumably by 2 : 1 fusion of the parental lines. Because no marker chromosomes were consistently present in either parent, we could not determine which parent contributed a double complement of chromosomes. Hybrid cells containing two genomes of one parent line and one genome of the other parent line have been previously described[2,7]. Five months after fusion the mean number of chromosomes in colony 1 was reduced from 130 to 111 and in colony 4 from 136 to 110. This loss of chromosomes is consistent with the results reported with other intraspecific hybrids[6,8].

Because they lack thymidine kinase, the parent 1T cells cannot incorporate exogenous thymidine into DMT. The hybrids incorporated 100 times more thymidine per μg of cell protein than the 1T cell and approximately half as much thymidine per μg of protein as the myeloma cell. We have found no reversion to thymidine kinase activity in more than 10^8 1T cells surveyed in GHAT medium.

The BALB/c mice from which the MPC 11 cell line was derived express surface antigens determined by the *H-2*^{d} allele, including H-2 specificity 31. H-2 specificity 5, not present on cells of *H-2*^{d} origin, was present on 1T, the parental fibroblast cell line of Swiss origin. We tested the parental lines and colonies 1 and 4 for the H-2 specificities 5 and 31 by examining the ability of serial dilutions of the cells to absorb cytotoxic activity from an antiserum-target cell detection system measuring each of these specificities[9,10]. 1T cells contained only specificity 5 and MPC 11 only specificity 31. The two hybrid colonies tested expressed both specificities. Similar findings have been reported by Spencer *et al.*[11] for the expression of H-2 antigen in other hybrids.

All hybrid colonies could not synthesize immunoglobulins, as shown by immunological precipitation of ^{14}C-labelled protein followed by polyacrylamide gel electrophoresis. After incubating the cells with ^{14}C-amino-acids for 5 min, 30 min, or 16 h, we examined cytoplasm[12] and secreted material. Part of each sample was analysed without immunological precipitation. Another portion was precipitated specifically with antiserum directed against H and L chains, and an equal portion was precipitated with a non-cross-reacting antiserum. Between 1 and 3% of the labelled protein of the hybrids precipitated nonspecifically. All samples were analysed by SDS acrylamide gel electrophoresis[13,14], in the presence of marker consisting of ^{3}H-labelled H and L chains secreted by the parent MPC 11 cells.

Our criterion for the synthesis of H or L chains was the presence, in the specific but not in the nonspecific precipitate, of a peak of ^{14}C-labelled material which electrophoresed identically with a marker protein. Figs. 1*a* and *b* show electropherograms of the cytoplasm and specifically precipitated cytoplasm of MPC 11 cells. The H and L chain peaks are clearly visible above the background of other cytoplasmic proteins (Fig. 1*a*). In Fig. 1*b*, this background has essentially been removed by the specificity of the immunologic precipitation. By contrast, the gel of the specifically precipitated hybrid colony 4 cytoplasm (Fig. 1*c*) shows no peak that coincides with the marker. A small peak of radioactivity that migrated one fraction ahead of the H chain marker, however, is seen in Figs. 1*c*, *d* and *f*. Specifically (Fig. 1*c*) and nonspecifically precipitated hybrid (Fig. 1*d*) cytoplasms appear identical. This shows that the hybrid does not synthesize immunoglobulin at levels comparable with MPC 11. To determine the limit of sensitivity of detection of H and L chain synthesis by this method, we mixed portions of the above MPC 11 and hybrid cytoplasmic preparations in an amount equal to that used in the gels shown in Figs. 1*b* and 2. The ratio of acid precipitable radioactivity

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was 6 : 1 in favour of the hybrid cytoplasm. The mixture was then specifically precipitated and an identical mixture non-specifically precipitated. Figs. 1*e* and *f* show the results from this reconstruction experiment. We found peaks that migrated identically with the H and L markers only in the specifically precipitated material (Fig. 1*e*). This showed that H and L chains could still be detected in the presence of a six-fold excess of radioactivity from the hybrid cytoplasm.

We performed a similar reconstruction experiment at a 50 : 1 hybrid to MPC 11 ratio (Fig. 2), but using hybrid cytoplasm labelled for 16 h. A peak that migrated identically with the L chain marker is again found only in the specifically precipitated sample which contains MPC 11 cytoplasm. After subtraction of cytoplasmic background, the number of counts in this peak is exactly that found in the L chain peak of an identical sample of MPC 11 cytoplasm analysed without the addition of hybrid cytoplasm (not shown). A similar, less prominent, but clearly identifiable peak is also found when a 100 : 1 ratio of counts was used, but is undetectable at a 500 : 1 ratio. This shows that we would have detected as little as 1% production of L chain compared with the myeloma parent. The detection of the immunoglobulin H chain is more difficult, because the minor peak of Figs. 1*c*, *d* and *f* is quite prominent here and migrates one fraction ahead of the H chain marker. The peak is seen both in the specifically and nonspecifically precipitated cytoplasm of hybrid colony 1 (Fig. 2), as well as in the 1T parent (Fig. 3). The presence of this non-immunoglobulin peak reduces the sensitivity of detection of H chain synthesis to about 4% of the level in the myeloma cells, as determined by interpolation between the results shown in Figs. 1 and 2.

If H or L chains were being synthesized and then degraded, they would be more readily detected after shorter labelling times. We therefore incubated colony 4 with radioactive amino-acids for 5 min and the cytoplasm was analysed as described above. The results were similar to those obtained

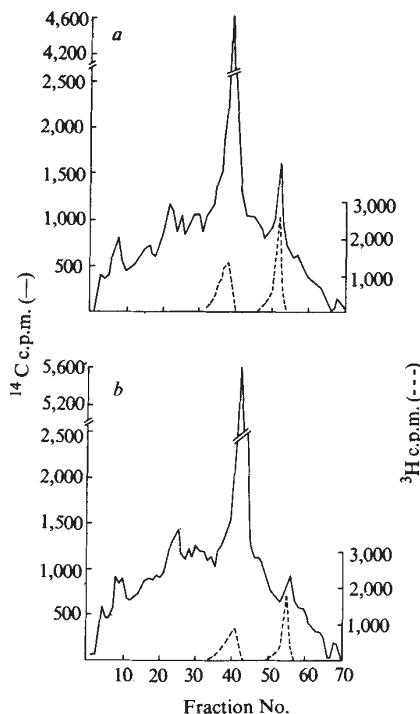

Fig. 2 Reconstruction experiment, 50 : 1 ratio. The experiment was performed exactly as in Fig. 1 except that colony 1 was labelled for 16 h in medium containing 10% dialysed foetal calf serum and 500,000 c.p.m. of colony 1 cytoplasm added to 10,000 c.p.m. of MPC 11 cytoplasm. *a*, Specifically precipitated; *b*, nonspecifically precipitated.

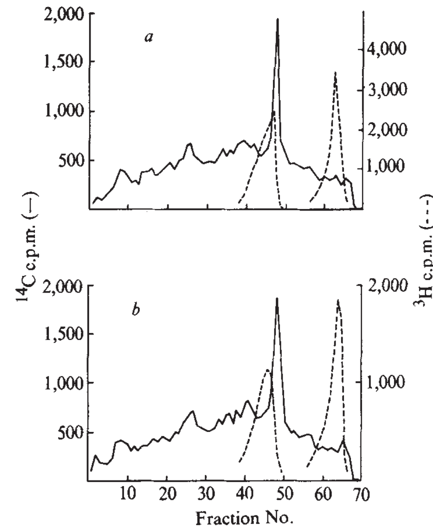

Fig. 3 Cytoplasm of 1T parent cells. 1T cells were labelled for 16 h; cytoplasm was immunologically precipitated and analysed as in Fig. 2. *a*, Specifically precipitated cytoplasm; *b*, nonspecifically precipitated cytoplasm.

after 30 min or 16 h of labelling. Colonies 1, 2, 3, 5 and 6 were also labelled for 30 min and the cytoplasm was specifically precipitated and analysed on gels. We found no peak of radioactivity that migrated with the marker.

The myeloma cells secrete only H and L chains. If the hybrids were synthesizing and secreting even small quantities of myeloma protein, the culture medium would be considerably enriched in these polypeptides. Colony 1 and colony 4 were therefore labelled for 16 h and the labelled protein in the culture medium was analysed as described for cytoplasm, with similarly negative results.

To determine whether a minor cell population had regained or retained the ability to make immunoglobulin, we examined the hybrids for H and L chains by immunofluorescence. The myeloma cells were strongly positive. 10^4 cells each of 1T, colony 1 and colony 4 were examined, and all were negative.

We have analysed six hybrids between a mouse myeloma and a mouse fibroblast cell. None synthesized immunoglobulin. The mechanism of this suppression is not clear. It is unlikely that chromosome loss leads to the cessation of immunoglobulin production because the hybrids were analysed before extensive loss of chromosomes and immunoglobulins were not produced by any of the six colonies. But the selective loss of chromosomes bearing structural or control loci for immunoglobulin cannot be ruled out. It is also possible that immunoglobulin producing hybrids were formed but did not attach to glass and were discarded. The GHAT selective medium did not directly suppress immunoglobulin synthesis, because MPC 11 continues to produce both H and L chain even after prolonged cultivation in that medium.

Spontaneous cessation of H chain synthesis in MPC 11 tissue culture cells occurs at the rate of 1.1×10^{-3}/cell/generation and loss of L chain synthesis at a rate at least ten times slower[15]. The two steps are sequential. Conversion of H+L chain producers to non-producers therefore proceeds at a rate of less than 10^{-7}/cell/generation. Formation of the type of hybrids isolated here suppresses both H and L chain production far more efficiently than does the spontaneous process in the parent MPC 11 line. Although it is clear that large amounts of MPC 11 H and L chains are not made by the hybrids, the assay used in these studies would not reveal very low levels of immunoglobulin production, especially if the immunoglobulin chains had lost their antigenicity.

Since this article was submitted for publication, somatic hybridization of immunoglobulin-producing cells has been

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reported by P. Periman (*Nature*, **228**, 1086; 1970) and B. Mohit and K. Fan (*Science*, **171**, 75; 1971).

P. COFFINO
B. KNOWLES
S. G. NATHENSON
M. D. SCHARFF

Albert Einstein College of Medicine,
1300 *Morris Park Avenue,*
Bronx, New York 10461

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Nucleotide Composition of RNA hybridized to Homologous DNA from Cells transformed by Avian Tumour Viruses

PART of the evidence which indicates that RNA tumour viruses replicate through a DNA intermediate[1] was the detection of DNA which is complementary to the viral RNA in leukaemic cells transformed by avian myeloblastosis virus (AMV)[2] and in cells transformed *in vitro* by avian sarcoma viruses, Schmidt-Ruppin (SR–RSV) and B–77 (ref. 3). If this DNA serves as a template for the viral RNA, it must be a copy of the entire viral genome. One of the necessary requirements for this function is that the homologous DNA has the same nucleotide composition as the viral RNA. In this study, the average base composition of the RNA which had been hybridized to homologous DNA from transformed cells was compared with the base composition of the input viral RNA. Two experimental conditions had to be met: (1) the recovery of all the ribonucleotides which had been hybridized, and (2) the absence of partially hybridized ribonucleotide sequences. The first requirement called for the deletion of the treatment of DNA–RNA hybrids with pancreatic ribonuclease fraction A and ribonuclease T_1 which had been used in our previous experiments because such a treatment can cause the non-random loss of hybridized nucleotides[4]. The second requirement called for a hybridization and washing procedure in which only specifically hybridized ribonucleotide sequences would remain bound to the filters. Both of these conditions were met by using fragmented viral RNA and a modified washing procedure which excluded the use of ribonuclease. The results show that the average nucleotide composition of the hybridized RNA is identical to that of the input viral RNA.

Schmidt-Ruppin-RSV, A classification, and AMV, BAI strain A, B classification, were used. For the work with SR–RSV, chick embryo fibroblasts were obtained from a line of White Leghorns (Kimber Farms SPF-K-137) which had been selected for its lack of infection by class A and B leukosis viruses. These embryos contain less than half the amount of DNA complementary to AMV–RNA that is present in normal K-137 embryos (Baluda, unpublished). DNA was extracted[2] from transformed fibroblasts 10 days after infection with SR–RSV at a multiplicity of 1 FFU per cell and after two subcultures. ^{32}P-labelled SR–RSV was obtained from the supernatants of similar transformed cells cultured in ^{32}P medium[5]. After 18 h of pre-labelling, fresh ^{32}P medium was added and the supernates were collected four times at 5 h intervals.

The preparation and purification of ^{3}H and ^{32}P-labelled AMV have been described[5,6]. The preparation of 71S–RNA from these purified viruses has also been described[5,6].

Table 1 demonstrates that with the use of ^{3}H–AMV–RNA fragmented into pieces of about 9S and with washing procedure 4, the non-specific binding of ^{3}H–RNA can be prevented without the use of ribonuclease. Mouse DNA which does not contain DNA homologous to the RNA of avian leukosis viruses[3] was used as the indicator of non-specific binding and bound only 2.3% as much ^{3}H–RNA as did leukaemic DNA. Furthermore, partial hybrids were not formed since the amount of ^{3}H–AMV–RNA that hybridized to leukaemic DNA was the same whether ribonucleases (pancreatic fraction A and T_1) were used or not. This procedure was used in the preparation of hybrids between transformed cell DNA and ^{32}P-labelled viral RNA.

Sixteen filters, each with about 50 μg of transformed cell DNA, were incubated in 2.5 ml. of hybridization mixture containing 8×10^5 c.p.m./ml. of ^{32}P-labelled 71S–SR–RSV–RNA and 3 mg/ml. of mouse RNA. Five mouse DNA filters, each with about 50 μg of DNA, were used as controls. After hybridization and washing, eight transformed cell DNA filters chosen at random and the five mouse DNA filters were counted to determine the amount of hybridized ^{32}P viral RNA. The same filters were recounted after elution of the hybridized RNA by boiling. As Table 2 shows, the mouse DNA filters bound less than 3% as much ^{32}P viral RNA per 100 μg of DNA as did transformed cell DNA. More than 87% of the ^{32}P RNA hybridized to homologous DNA was eluted by boiling in 0.1 SSC whereas only 35% of the non-specifically bound radio-

Table 1 Comparison of Various Washing Procedures to remove Non-specifically Hybridized Viral RNA

Procedure	RNAase treatment A (μg)	RNAase treatment T_1 (units)	CPM hybridized per 100 μg DNA from Myeloblasts	CPM hybridized per 100 μg DNA from Mouse
1	25	10	984 ± 25 (4)	10 ± 1 (4)
2	2.5	5	963 ± 31 (4)	Not done
3	1.0	—	948 ± 97 (4)	28 ± 4 (5)
4	—	—	993 ± 34 (4)	23 ± 4 (5)

The preparation of ^{3}H-labelled 71S–RNA from purified AMV has been described before[5,6]. The 71S viral RNA was fragmented into pieces of about 9S (130,000 daltons) by boiling in 0.1 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M Na citrate) for 5 min followed by sonication for 5 min at 20,000 c.p.s. with a Branson sonifier, model S-110. Hybridization was carried out, as previously described[3], in two vials containing either sixteen leukaemic DNA filters or sixteen mouse DNA filters in 3 ml. of hybridization mixture: 1.04×10^6 c.p.m./ml. of fragmented ^{3}H-labelled 71S–AMV–RNA (specific activity 5×10^5 c.p.m./μg), 2.6 mg/ml. of mouse RNA and 0.05% SDS in 4 × SSC. DNA was extracted, purified, denatured and trapped on 'Millipore' filters as previously described[2]. Non-hybridized ^{3}H–RNA was removed by either one of four procedures: (1) Baluda[3]; (2) Soeiro and Darnell[7]; (3) same as (4) plus RNAase A (1 μg/ml.); (4) the filters were agitated three successive times in 300 ml. of 2 × SSC at 70° C, kept at 75° C in 2 × SSC for 30 min, placed on 'Millipore' filter holders and each side of the filter was rinsed by suction with 75 ml. of 2 × SSC at 70° C. The rim of the filters was cut off and then the filters were washed three successive times with 100 ml. of 2 × SSC containing 0.5% sodium dodecyl sulphate (SDS) at 70° C. This was followed by one final rinse in 300 ml. of warm 2 × SSC without SDS. Mean c.p.m. ± standard deviation and in parentheses the number of filters used. Background of 60 c.p.m. has been subtracted.

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