

Table 2 Specific binding of ^3H -oestradiol to intact and operated mammary glands of unilaterally thelectomised rats

Rats	^3H -oestradiol bound (fmol per mg cytosol protein*)		Significance
	Intact mammary gland	Thelectomised mammary gland	
Virgin ($n = 4$)	10.8 ± 5.1	9.25 ± 4.75	NS
Early pregnancy (1–11 days) ($n = 6$)	6.7 ± 5.4	10.2 ± 10.3	NS
Late pregnancy (12–22 days) ($n = 9$)	4.9 ± 4.1	4.3 ± 5.7	NS
Early lactation (2–7 days) ($n = 6$)	8.7 ± 4.1†	4.7 ± 3.8	NS
Late lactation (8–21 days) ($n = 7$)	20.9 ± 15.1	5.0 ± 3.7	$P < 0.02$
Weaned (5–12 days) ($n = 6$)	8.6 ± 4.1	3.2 ± 3.1	$P < 0.03$

Oestradiol binding was assayed in the cytosol fraction (100,000 g supernatant). Cytosol plus buffer (500 μl) was incubated overnight (5 °C) with saturating amounts (2 pmol) of [6,7- ^3H]oestradiol-17 β (44 Ci mol $^{-1}$, Radiochemical Centre, Amersham) in the absence and presence of unlabelled oestradiol (2 nmol). Unbound steroid was removed by incubation at 0 °C for 15 min with dextran-coated charcoal (1 ml 0.5% activated charcoal, Sigma, 0.05% dextran T80, Pharmacia). Charcoal was precipitated by centrifugation (1,500g for 10 min) and the supernatants decanted into 10 ml Instagel scintillation phosphor (Packard) and the radioactivity determined using a Packard TriCarb B2450 liquid scintillation counter, with automated external quench correction. The binding was specific for oestrogens being displaced by oestradiol and diethylstilboestrol, but not by testosterone, dexamethasone, cortisol or progesterone. Values are means \pm s.d.; n = no. of observations; NS, not significant, $P > 0.05$.

* Protein measured by a modified Lowry method¹⁸.

† $P = 0.03$ versus mammary gland in late pregnancy.

When teats are blocked, ligated or removed, milk accumulates in the mammary gland. Early changes depend on whether the litter remains with the mother. If the litter is removed, then even in intact glands milk accumulates and mammary blood flow falls within 8 h as a result of reduced cardiac output and a reduced proportion of cardiac output being taken by the mammary gland¹⁵. If, as in the present experiments, suckling continues but milk removal is prevented, then even more milk collects in the tissue but the blood flow does not fall¹⁵. However, by 36–48 h capillary closure occurs in the mammary gland whether suckling occurs or not⁵, and there is marked reduction in metabolic activity^{1–3}. The collapse of the capillary bed can be seen by blanching of the tissue and was further demonstrated by Silver⁵ by failure of the gland to give a milk ejection response to oxytocin administered intravenously, whereas a response could still be obtained if oxytocin was applied locally to the exposed surface of the gland. Capillaries remained empty beyond 120 h in suckled glands⁵. As involution progresses, the proportion of cardiac output going to the mammary gland falls, apparently regulated locally by depressed mammary activity^{15,16}.

Our results indicate the importance of blood supply and rate of delivery of hormones to the tissue in the control of receptor levels. This could be of considerable importance in the regulation of receptor levels in breast cancer tissue, since angiogenesis, blood vessel development, is stimulated by neoplastic cells¹⁷.

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1. McLean, P. *Biochem. J.* **90**, 271–278 (1964).

2. Jones, E. A. *Biochim. biophys. Acta* **177**, 158–160 (1968).

3. Hamosh, M., Clary, T. R., Chernick, S. S. & Scow, R. O. *Biochim. biophys. Acta* **210**, 473–482 (1970).

4. Lyons, W. R. *Proc. R. Soc.* **B149**, 303–325 (1958).
5. Silver, I. A. *J. Physiol., Lond.* **133**, 65–66P (1956).
6. Hayden, T. J., Bonney, R. C. & Forsyth, I. A. *J. Endocr.* **80**, 259–269 (1979).
7. Holcomb, H. H., Costlow, M. E., Buschow, R. A. & McGuire, W. L. *Biochim. biophys. Acta* **428**, 104–112 (1976).
8. Smith, R. D., Hilf, R. & Senior, A. E. *Cancer Res.* **36**, 3726–3731 (1976).
9. Bohnet, H. G., Gomez, F. & Friesen, H. G. *Endocrinology* **101**, 1111–1121 (1977).
10. Gardner, D. G. & Whitliff, J. L. *Biochim. biophys. Acta* **320**, 617–627 (1973).
11. Hsueh, A. J. W., Peck, E. J. & Clark, J. H. *J. Endocr.* **58**, 503–511 (1973).
12. Leung, B. S., Jack, W. M. & Reiney, C. G. *J. Steroid Biochem.* **7**, 89–95 (1976).
13. Forsyth, I. A. & Hayden, T. J. *Symp. Zool. Soc. Lond.* **41**, 135–163 (1977).
14. Leung, B. S. & Sasaki, G. H. *Biochem. biophys. Res. Commun.* **55**, 1180–1187 (1973).
15. Hanwell, A. & Linzell, J. L. *J. Physiol., Lond.* **233**, 111–125 (1973).
16. Chatwin, A. L., Linzell, J. L. & Setchell, B. P. *J. Endocr.* **44**, 247–254 (1969).
17. Gullino, P. M. *J. natn. Cancer Inst.* **61**, 639–643 (1978).
18. Hartree, E. F. *Analyt. Biochem.* **48**, 422–427 (1972).

Novel expression-linked copies of the genes for variant surface antigens in trypanosomes

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Pathogenic African trypanosomes evade the immune system of their mammalian hosts by the sequential expression of alternative cell-surface glycoproteins (reviewed in refs 1, 2). Variant surface glycoproteins (VSGs) purified³ from cloned variants of *Trypanosoma brucei* have similar molecular weights (about 60,000), but differ in amino acid composition³, N-terminal amino acid sequence⁴ and C-terminal structure⁵. We have cloned DNA complementary to the messenger RNAs for four immunologically distinct VSGs⁶ and hybridised these complementary DNAs (cDNAs) with restriction digests of *T. brucei* nuclear DNA, fractionated by gel electrophoresis and transferred to nitrocellulose strips. Each cDNA recognises a unique set of fragments and this basic set is present unaltered in the nuclear DNAs from the four variants. In addition, each probe recognises an extra fragment only in nuclear DNA isolated from cells expressing the VSG corresponding to the cDNA probe. We infer that activation of a VSG gene involves the production of an expression-linked copy of that gene.

The cDNA clones used in our experiments were originally identified⁶ on the basis of their ability to hybridise only to poly(A)⁺ RNA from the homologous variant and this specificity is further documented in Fig. 1. Total poly(A)⁺ RNA from all four variants of *T. brucei*, strain 427, was size-fractionated by agarose gel electrophoresis (Fig. 1a), covalently linked to paper⁷ and then hybridised with one of the four cloned cDNAs. Figure 1b shows that cDNA of variant 117 only hybridises with homologous RNA; the same was found with the other three cDNAs (not shown). The mobility of the RNA species that hybridise is variant dependent (Fig. 1c), the calculated size varying from 2,250 nucleotides (variant 117) to 1,950 nucleotides (variant 221)⁶. This variation correlates with the apparent molecular weights of the corresponding pre-VSGs, deduced from their mobilities in SDS polyacrylamide gels. These decrease in the same order, 117 being 62,000, 221 being 52,000 (ref. 6).

The genes corresponding to these VSG-specific cDNAs were analysed by the Southern blotting technique⁸. Nuclear DNA from one *T. brucei* variant was digested with various restriction endonucleases, size-fractionated by agarose gel electrophoresis, blotted on to nitrocellulose strips and hybridised with each of the four cDNAs. Figure 2 shows results with three of these. Each

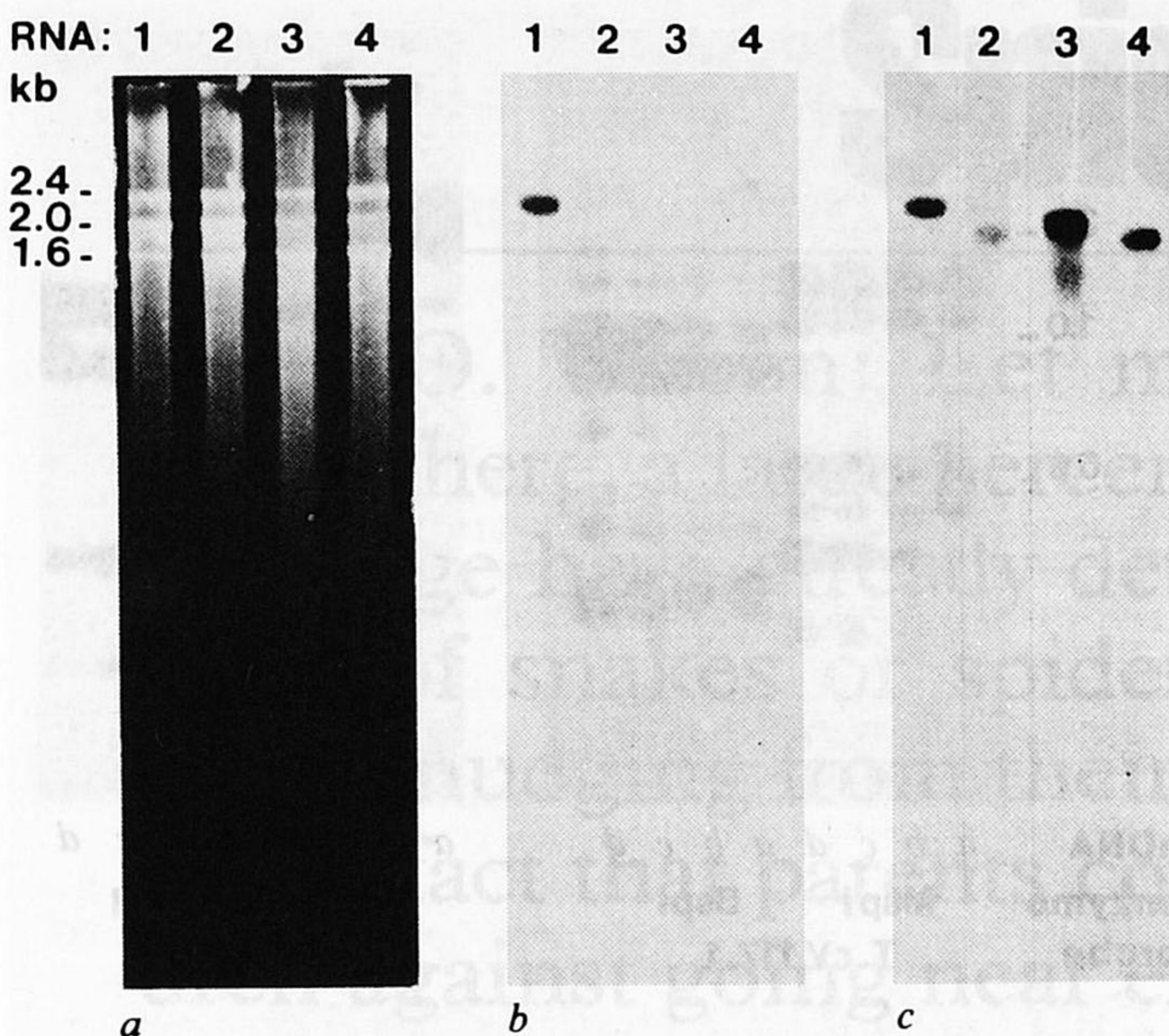


Fig. 1 Hybridisation of cloned cDNA with poly(A)⁺ RNA from the homologous variant and other variants. Glyoxal-treated poly(A)⁺ RNA (2 µg) from each of the four trypanosome variants was electrophoresed through a 1.75% agarose gel in Tris-borate buffer¹⁶. After transfer of the RNA to diazobenzoxymethyl paper⁷, the filters were hybridised with nick-translated recombinant plasmid DNA containing sequences complementary to VSG mRNA⁶. *a*, Photograph of the ethidium-stained gel after electrophoresis; lane 1, variant 117 RNA; lane 2, 118 RNA; lane 3, 121 RNA; lane 4, 221 RNA. The large rRNA contains an internal break and yields two bands. *b*, Autoradiogram of the RNA in *a* after transfer to diazobenzoxymethyl paper and hybridisation with a recombinant plasmid containing DNA complementary to VSG 117 mRNA (T-cV117-1). *c*, As *b*, but each RNA hybridised with the homologous cDNA probe; lane 1, T-cV117-1; lane 2, T-cV118-2; lane 3, T-cV121-3; lane 4, T-cV221-12. (Figure assembled from different replica gels.) The recombinant plasmids were made by inserting duplex DNA complementary to VSG mRNA into the *Pst*I site of plasmid pBR322 by the dG·dC tailing technique⁶. The sizes of the inserts are (in base pairs): T-cV117-1, 820; T-cV118-2, 1,500; T-cV121-3, 800; T-cV221-12, 1,200. See ref. 6 for isolation of poly(A)⁺ RNA, plasmid isolation, ³²P labelling of DNA by nick-translation, gel handling and other evidence that the cDNA inserts of these four plasmids have no sequence homology under standard hybridisation conditions.

cDNA hybridises to a different set of bands, confirming that each probe 'sees' different genes, as anticipated from Fig. 1. The 118 and 121 probes hybridise mainly to one or two bands in each digest, indicating that they primarily recognise one gene. At long exposures other faint bands become visible, suggesting that the probes cross-hybridise to other genes that show partial cross-homology with the 118 and 121 genes. The 117 cDNA probe hybridises to many different bands in all digests. We attribute this to extensive cross-hybridisation to a family of related VSG genes, because more stringent washing of the hybrids (in 0.1 × SSC at 60 °C) removes the label from all but a few of these bands (not shown). An even more complex pattern has been found with the 221 cDNA (not shown).

There are no differences between the *Hap*II and *Msp*I digests for any of the probes. Both enzymes recognise the sequence CCGG, but cleavage by *Hap*II is prevented by methylation of the C in the CpG doublet, whereas cleavage by *Msp*I is not⁹. Partial methylation of C in the CpG sequence, therefore, does not complicate the digests shown here.

To determine whether expression of a VSG gene results in sequence alterations in or around that gene, we compared the

hybridisation of each cDNA probe with homologous and heterologous nuclear DNA digests. Figure 3 shows results with the 118 cDNA probe. In each digest this probe sees one DNA fragment more in the homologous 118 nuclear DNA than in the heterologous 117 DNA. We attribute the fragments common to the 117 and 118 DNA digests to a basic copy of the 118 VSG gene, present in both variants. The results of double digestion experiments with two restriction enzymes fit a *Pst*I × *Eco*RI × *Bam*HI × *Hind*III map of this basic copy which accounts for all

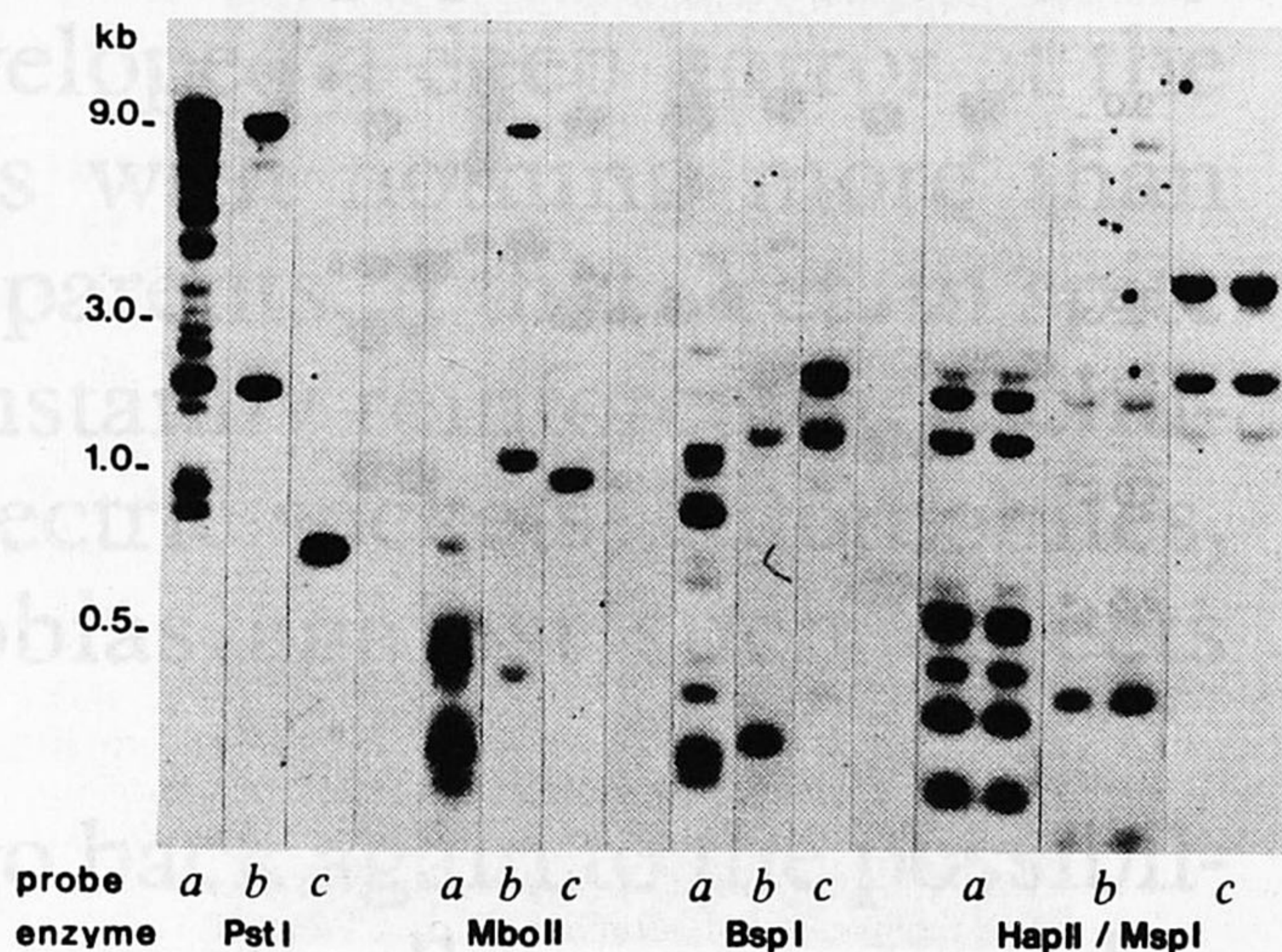


Fig. 2 Autoradiogram of variant-specific cDNA probes hybridised to restriction enzyme digests of nuclear DNA from *T. brucei* variant 118. Nuclear DNA was isolated by a modification of the kinetoplast DNA isolation procedure¹⁷, digested in standard conditions¹⁷ with the enzyme indicated and electrophoresed through a 0.6% agarose gel¹⁷. The gel was blotted on to nitrocellulose filter strips as described elsewhere⁸ and hybridised with nick-translated DNA from the recombinant plasmids indicated. See Fig. 1 legend for further details. Probes: *a*, T-cV117-1; *b*, T-cV118-2; *c*, T-cV121-3.

fragments seen in blots without invoking intervening sequences or the presence of more than one basic 118 VSG gene. In each digest shown in Fig. 3 the 118 cDNA probe hybridises to one extra band in the homologous 118 nuclear DNA, not present in the 117 nuclear DNA. An extra band was also found in the *Mbo*II, *Hap*II and *Msp*I digests (not shown). We attribute these extra bands to an additional expression-linked copy of the 118 VSG gene only present in 118 nuclear DNA.

Analogous results were obtained with the 117 and 121 cDNA probes. Figure 4 shows that the fragments hybridising with the 117 cDNA are identical except for an extra band in the *Msp*I digest of the homologous 117 nuclear DNA. Extra bands only in homologous DNA were also observed in the *Hap*II, *Eco*RI and *Pst*I digests (not shown), but not in *Bsp*I (Fig. 4) and *Mbo*II digests (not shown). The fact that an extra band does not appear in all digests of homologous DNA is not surprising. If the sequences around a gene change, this will only result in altered fragments in blots if the enzyme used cuts far enough from the gene segment recognised by the probe to include the altered sequence. The results with the 121 cDNA are more complex. In this case extra bands are observed in both the 121 and the 221 nuclear DNAs, but the extra bands are not identical in size. It is of interest that variant 221 has arisen during *in vitro* culture from variant 52, which is immunologically identical to variant 121 (ref. 10). The extra band in the 221 digest could, therefore, represent an inactivated form of the expression-linked copy of the 121 gene, but this is only one of several possibilities.

Analysis of several blots like those shown in Fig. 3 has yielded no reproducible differences in the relative intensities of the

bands attributable to the basic 118 VSG gene in variant 117 and variant 118 DNA. Also, the extra bands hybridise at approximately the same intensity as the bands from the basic copy, allowing for a less efficient transfer of large bands in blotting experiments. Our results, therefore, suggest that the expression-linked copy is an extra copy of the 118 VSG gene. This would

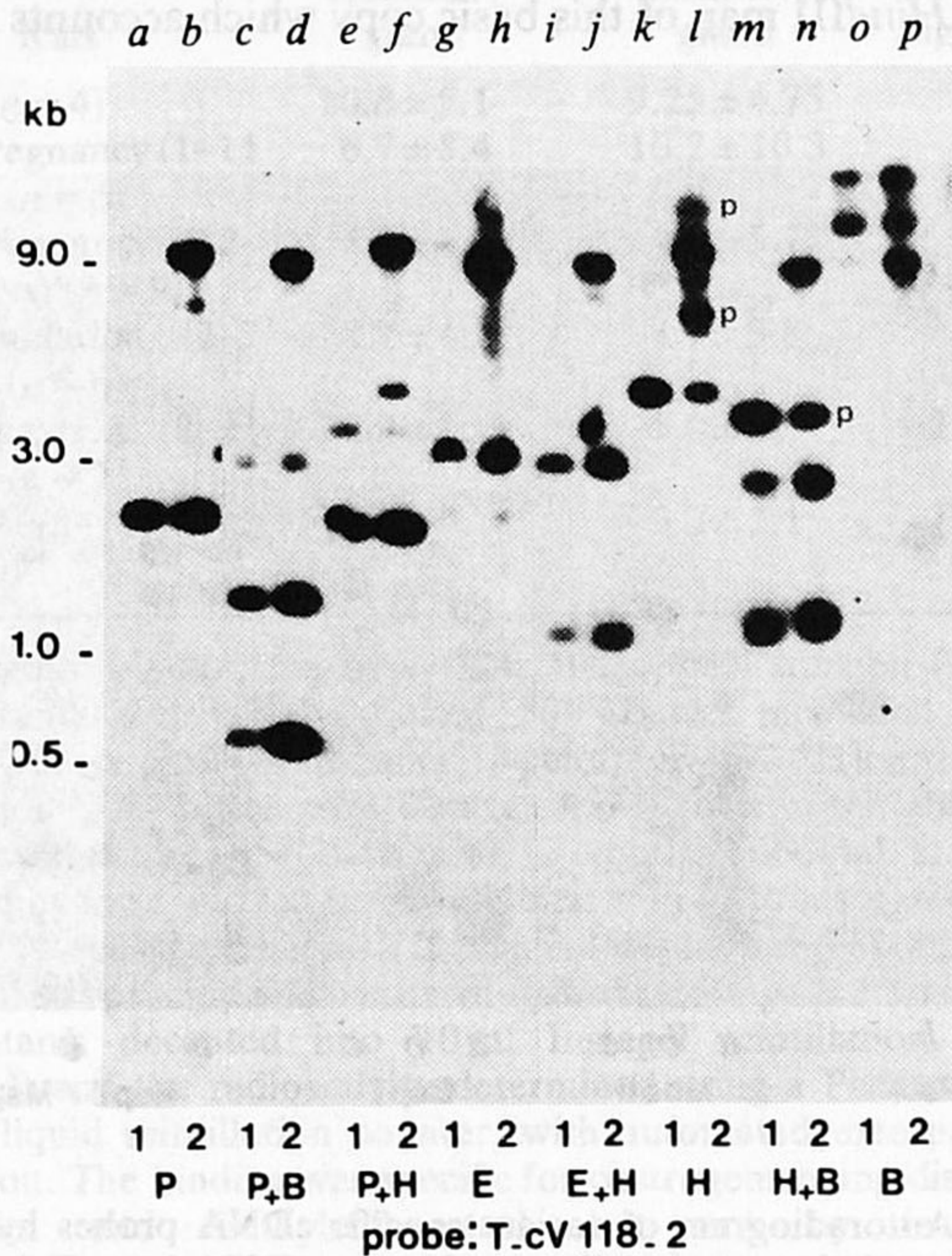


Fig. 3 Hybridisation of DNA complementary to VSG 118 mRNA to nuclear DNA digests of variants 117 and 118. The restriction digests were electrophoresed through a 0.7% agarose gel. Further conditions as in Fig. 2. The p marks persistent partial *HindIII* digestion products, absent in other experiments. Lane 1, nDNA 117; lane 2, nDNA 118; P, *PstI*; B, *BamHI*; H, *HindIII*; E, *EcoRI*.

imply the presence of minimally two basic and two expression-linked copies of this gene per nucleus, if trypanosomes are diploid. The ploidy of *Trypanosoma* is not known, however, and the available data on DNA complexity (A.C.C.F. and P. B., unpublished) and the amount of DNA per cell¹¹ are so imprecise that they are compatible with anything between haploid and tetraploid.

To account for the results of the blotting experiments, the formation of the expression-linked copy must be associated by a reshuffling of DNA sequences which alters the neighbouring sequences. This reshuffling could either move the gene into a new (expression) site¹²⁻¹⁴, as in the cassette mechanism for mating-type switching in yeast¹⁴, or an insertion sequence could be put next to the extra copy¹⁵. Whatever the mechanism, the sequences flanking the expression-linked copy of the VSG 118 gene are unusual in that at one side no *PstI*, *HindIII*, *BamHI* or *EcoRI* site is present within 8 kilobase pairs of the gene. It is interesting that large extra bands have also been observed when the 117 cDNA is hybridised with 117 nuclear DNA cleaved with *MspI* (Fig. 4), *PstI* or *EcoRI*. The flanking sequence, may, therefore, be similar whatever alternative gene is being expressed. The simplest interpretation of our results is that the expression-linked copy is the one transcribed and that the novel flanking sequences are responsible for this; however, this remains to be confirmed.

The recombination event that we expect to be responsible for the positioning of the extra copy in the postulated expression site could easily be accompanied by further recombination events that increase sequence diversity in the VSG genes. There is no

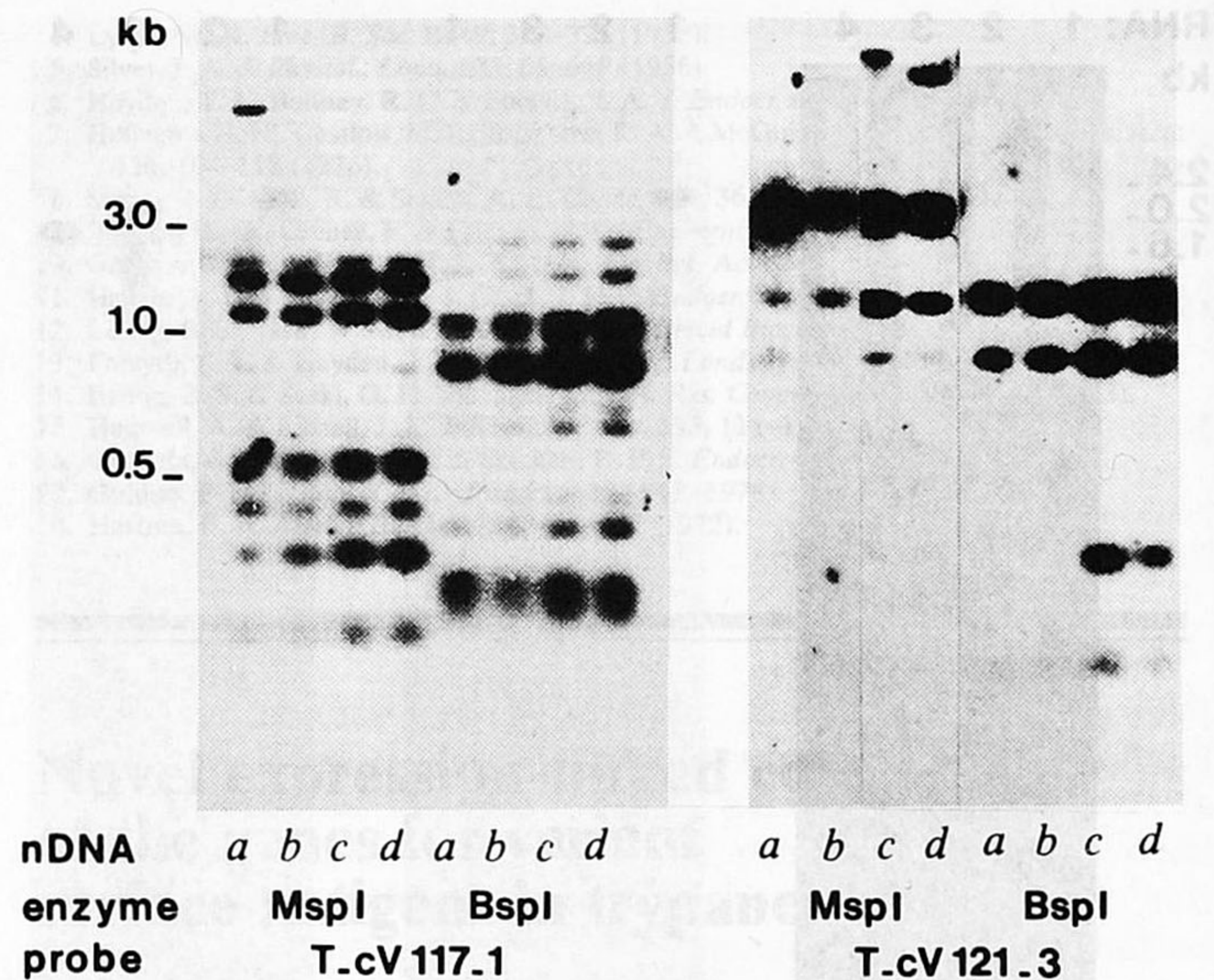


Fig. 4 Autoradiogram showing hybridisation of VSG-specific cDNA probes with homologous and heterologous nuclear DNAs. Nuclear DNA from the four variants indicated was electrophoresed through a 1.2% agarose gel; see Fig. 2 legend for further details. nDNA: a, 117; b, 118; c, 121; d, 221.

evidence for this either in previous work on VSGs (refs 2-5 and G.A.M.C., unpublished) and their genes⁶, or in our present experiments, as the expression-linked copy is not recognised by the heterologous probes tested. It seems likely, therefore, that antigenic diversity in trypanosomes is genomic and finite and not created by a clever reassortment of a limited number of DNA sequences to yield an unlimited number of VSG genes. It is possible, however, that the novel flanking sequence in the expression site provides not only a promoter but also the 5'-leader sequence and signal peptide (if present) for each VSG. This can be tested when genomic clones are available; work is in progress to obtain them.

Williams and coworkers¹⁸ have also observed rearrangement of DNA associated with expression of another line of *T. brucei*. In their case, however, there is no clear conservation of bands associated with a basic copy and, moreover, there are also alterations in another clone apparently not associated with expression. We cannot account for this crucial difference from our results.

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- Vickerman, K. *Nature* **273**, 613 (1978).
- Cross, G.A.M. *Proc. R. Soc. B* **202**, 55 (1978).
- Cross, G.A.M. *Parasitology* **71**, 393 (1975).
- Bridgen, P. J., Cross, G. A. M. & Bridgen, J. *Nature* **263**, 613 (1976).
- Johnson, J. G. & Cross, G. A. M. *Biochem. J.* **178**, 689 (1979).
- Hoeijmakers, J. H. J., Borst, P., Van den Burg, J., Weissmann, C. & Cross, G. A. M. *Gene* **8**, 391 (1980).
- Alwine, J. C., Kemp, D. J. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5350 (1977).
- Southern, E. M. *J. molec. Biol.* **98**, 503 (1975).
- Waalwijk, C. & Flavell, R. A. *Nucleic Acids Res.* **5**, 3231 (1978).
- Doyle, J. J., Hirumi, H., Hirumi, K., Lupton, E. N. & Cross, G. A. M. *Parasitology* (in the press).
- Riou, G. & Pautrizel, R. *J. Protozool.* **16**, 509 (1969).
- Kamp, D., Kahmann, R., Zipser, D., Broker, T. R. & Chow, L. T. *Nature* **271**, 577 (1978).
- Zieg, J., Hillmen, M. & Simon, M. *Cell* **15**, 237 (1978).
- Strathern, J. N., Newlon, C. S., Herskowitz, I. & Hicks, J. B. *Cell* **18**, 309 (1979).
- Bukhari, A. I., Shapiro, J. A. & Adhya, S. L. (eds) *DNA: Insertion, Elements, Plasmids, and Episomes* (Cold Spring Harbor Laboratory, New York, 1977).
- Van Ommen, G. J. B., Groot, G. S. P. & Grivell, L. A. *Cell* **18**, 511 (1979).
- Borst, P. & Fase-Fowler, F. *Biochim. biophys. Acta* **565**, 1 (1979).
- Williams, R. O., Young, J. R. & Majiwa, P. A. O. *Nature* **282**, 847 (1979).