Isolation and characterization of multiple human genes homologous to the oncogenes of avian erythroblastosis virus

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Human DNA sequences complementary to the oncogenes v-erbA and v-erbB of avian erythroblastosis virus have been isolated from a genomic DNA library. Two clones, λhe-A1 and λhe-A2, were related to the erbA gene and one to the erbB gene (λhe-B). The two erbA genes were only distantly related to each other as judged from hybridization analysis. Furthermore, human chromosomal DNA appears to contain one or two additional genes analogous to the λhe-A2 sequence, whereas the mouse genome contained only two genes complementary to λhe-A1 and λhe-A2, respectively. Poly-adenylated RNA species, 5.0 kb in size, were found in the human HeLa and the human hematopoetic K562 cell lines, suggesting that at least some of the erb-related genes are active and do not represent pseudogenes. Taken together, the data demonstrate that two distantly related classes of erbA genes exist in human and mouse DNA, and that multiple copies of genes belonging to one of these two classes exist in the human genome.
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Introduction

The avian erythroblastosis virus (AEV) induces sarcomas and erythroblastosis in infected birds, and the virus transforms fibroblasts and erythroblasts in vitro (for review, see Graf and Beug, 1978). The virus carries two host cell-derived genes, denoted v-erbA and v-erbB, of which the latter encodes the transforming capacity of the virus (Frykberg et al., 1983). However, mutants of AEV defective in erbA induce an atypical erythroblastosis in infected chickens, characterized by the presence in peripheral blood of more mature erythroid progenitor cells than found in erythroblastosis induced by wild-type AEV. It has therefore been suggested that erbA enhances the oncogenic effects of erbB while being unable to induce neoplasm independently (Frykberg et al., 1983).

The erbA gene encodes a 75-K gag-erbA fusion protein (Hayman et al., 1979), whereas the erbB encodes a phosphorylated membrane glycoprotein of mol. wt. ~ 68 000 (Privalsky et al., 1982; Hayman et al., 1983). No enzymatic functions have so far been associated with the v-erb gene products.

The v-erb genes originate in the chicken genome; the c-erb genes are independently transcribed into multiple mRNAs (Vennström and Bishop, 1982) and their expression is modulated in chicken tissues (Gonda et al., 1982). These genes have been reported to be located on small (or micro-) chromosomes of chicken (Wong et al., 1981). However, it is not known if the two erb-genes are linked.

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Roussel et al. (1979) previously demonstrated that the human genome contains sequences homologous to the onco-genes of AEV. We have isolated several molecular clones containing human sequences complementary to the erbA and the erbB genes. Analysis of these clones suggest that the human genome contains at least one gene homologous to erbB and three different genes homologous to erbA. At least two members of the human erbA gene family are transcribed into discrete species of RNA, suggesting that they are actively expressed.

Results

Isolation of human erbA and erbB sequences

A human genomic DNA library established with lambda phage Charon 4A was screened with probe 2 (described in Figure 1A) which is representative for the two erb domains of the viral genome. Three different clones were isolated and analyzed by restriction enzyme digestion and filter hybridization with the probes 1—6 specific for defined regions in the erb-genes. EcoRI fragments of two clones, λhe-A1 (Figure 1B) and λhe-A2 (Figure 1C), hybridized with probes 1, 2, 3 and 4, but not with probes 5 and 6, demonstrating that they encode erbA sequences (the results with probe 6 are not shown). The third clone, λhe-B, hybridizes with probes 1 and 2 (representative for both v-erb domains) and with probe 5, which is erbB specific (Figure 1D). No hybridization with the erbB-specific probes 4 and 6 was detected under even less stringent hybridization conditions (data not shown). The λhe-B clone could not be investigated further because it contained multiple repetitive sequences. The hybridization results are summarized in Figure 1E. The polarity of the erb sequence in λhe-A1 was deduced from the differential hybridization of probes 1 and 2: probe 1 encodes sequences from the 5' end of the erbA not present in probe 2, and it hybridizes with a 6.0-kb EcoRI-HindIII fragment of λhe-A1, which is unreactive with the other probe. The polarity of the λhe-A2 clone was deduced from the hybridizations with probes 3 and 4: a 2.0-kb EcoRI fragment anneals with probe 3 (representative for the middle portion of the erbA) and a 4.0-kb fragment with probe 4, which encodes the 3' part of the erbA.

These results did not unambiguously establish that the clones λhe-A1 and λhe-A2 only encode erbA sequences, since probe 4 is also reactive with the 5' portion of the erbB. Therefore, the clones of lambda DNA were labeled in vitro and hybridized to filters carrying SacI and PstI fragments of cloned AEV DNA. Figure 2 shows that both human erbA clones only hybridized with the 2.1-kb fragment representative for v-erbA, and not with the 1.8-kb erbB fragment (see Figure 1A for locations of fragments on the AEV genome), indicating that the clones only contain erbA sequences. In addition, the λhe-A1 clone anneals with three PstI fragments of 0.5, 1.5 and 2.1 kb from v-erb, whereas the λhe-A2 clone anneals only with the 0.5-kb and 2.1-kb fragments (Figure 2). The absence of hybridization of the λhe-A2 clone with the 1.5-kb fragment, which is located in the 5' part of the v-erbA, and the weaker hybridizations to the 2.1-kb fragment, indicate that the erb sequence in λhe-A2 has diverged more
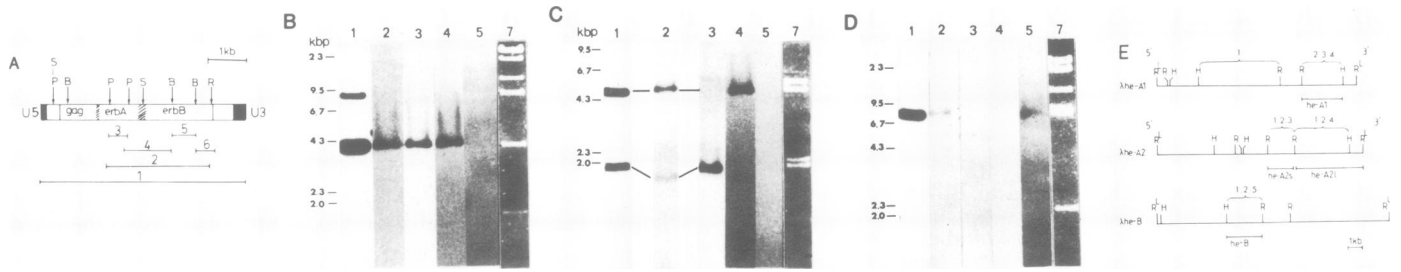
Fig. 1. Restriction enzyme analysis of lambda clones containing human *erb* sequences. Lambda DNAs were digested with *Eco*RI, the resulting fragments were separated in an agarose gel and transferred to nitrocellulose filters. Immobilized fragments were hybridized with the probes 1−6 derived from the AEV genome as shown in **panel A. Panel B:** λhe-A1 DNA; **panel C:** λhe-A2 DNA; **panel D:** λhe-B DNA; **panel E:** restriction endonuclease cleavage maps of the inserts in the lambda phages. The brackets indicate fragments reactive with the viral probes 1−6 described in **panel A.** The bars below the individual restriction maps indicate the framents used as probes in subsequent experiments. R: *Eco*RI; H: *Hind*III; R^L: *Eco*RI sites generated by the addition of synthetic linkers used for establishing the library. Some of the ethidium-bromide stained DNA bands have been lost in the reproduction processes.
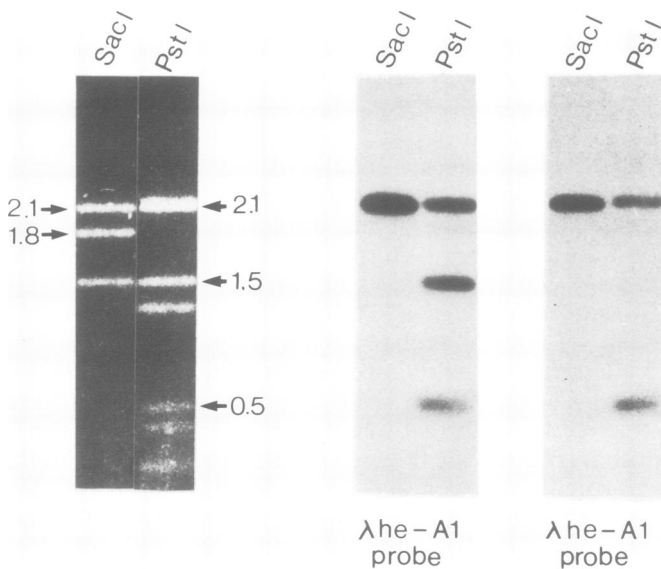

Fig. 2. Hybridization of human *erbA* sequences to AEV DNA. AEV DNA cloned in the *Eco*RI site of pBR313 was digested first with *Eco*RI and then with *Sac*I or *Pst*I. The fragments were separated in agarose gel, transferred to a nitrocellulose filter and hybridized with nick-translated λhe-A1 or λhe-A2 DNA. The fragments indicated by arrows encode viral *erb* specific sequences.

Fig. 3. Relatedness between human *erbA* sequences. **Panel A:** λhe-A1 or λhe-A2 DNAs were cleaved with *Eco*RI, fragments were separated in an agarose gel and transferred to a nitrocellulose filter. Cross-hybridizations were carried out with the he-A1 probe (see Figure 1) or with the entire λhe-A2 probe. A1 = λhe-A1; A2 = λhe-A2. **Panel B:** the *erb*-specific 4.8-kb *Eco*RI fragment in λhe-A1 was subcloned in pBR322; this DNA was cleaved with *Eco*RI and *Hind*III and the resulting fragments were separated in an agarose gel and transferred to a nitrocellulose filter for hybridization analysis. **Lane 1:** EtBr-stained DNA; **lane 2:** hybridization with λhe-A2 DNA; **lane 3:** hybridization with nick-translated human placental DNA; **lane 4:** hybridization with *v-erbA* + B probe (probe 2).

from *v-erbA* than λhe-A1. However, the possibility exists that λhe-A2 does not contain the whole locus. We considered this unlikely, since 7.3 kb of DNA precedes the *erb* sequences in this clone (Figure 1E), which is much larger than an average intron. In addition, the chicken *erbA* gene has no introns larger than 1 kb (Vennström and Bishop, 1982).

The human erbA sequences comprise a family of genes

Cross-hybridization experiments between the human *erbA* clones were carried out to investigate the relatedness between the genes. The restriction fragments of the λ clones that were used as probes in some of the experiments are indicated by bars in the restriction maps in Figure 1E. The hybridization of an *erbA1*-specific probe (designated he-A1; its location is indicated in Figure 1E) to fragments of λhe-A1 and λhe-A2 are shown in Figure 3A: only weak hybridization is observed to two λhe-A2 fragments as compared with the homologous annealing. The *v-erb* A + B probe also anneals with the two λhe-A1 fragments (see Figure 1), indicating that the observed hybridization is due to homologous *erb* sequences. The *erbA2*-specific probe (he-A2l; it represents a 3′ portion of the
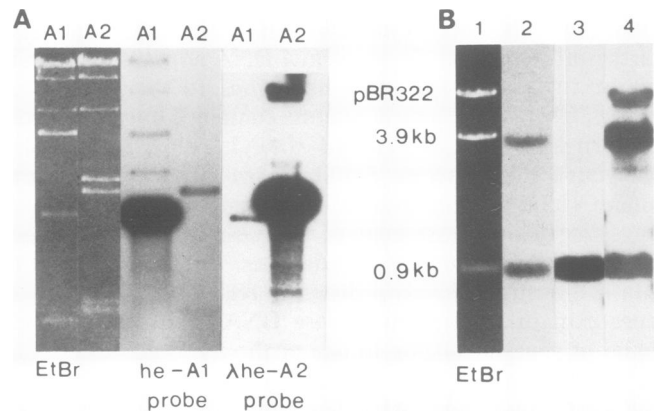
human gene as shown in Figure 1E) also anneals with the *erb*-specific fragment of λhe-A1 in a cross-hybridization experiment (Figure 3A); but again, the cross-reactivity is low when compared with the homologous annealing. To show that the cross-hybridization is due to homologous *erb* sequences and not to human repetitive sequences common to both λ clones, nick-translated total human DNA was annealed to a subclone of the λhe-A1 DNA. This clone contains the 3.9-kb *Eco*RI-*Hind*III fragment used as the he-A1 probe and an adjacent 0.9-kb *Hind*III-*Eco*RI fragment located in the 3′ portion of the insert in the λhe-A1 DNA. Lane 3 in Figure 3B shows that the 0.9-kb fragment strongly reacts with the nick-translated human DNA, whereas the 3.9-kb fragment does not contain repetitive sequences. In addition, lanes 2 and 4 show that both fragments reacted with labeled λhe-A2 DNA and *v-erb* probe (probe 1). Taken together, the results demonstrate that the hybridization observed between the λhe-A1 and λhe-A2 clones is due most likely to complementary *erb* sequences and not to the presence of human repetitive elements common to both DNAs. In addition, the experiments show that the human *erb* genes have diverged significantly from each other as judged by the moderately stringent annealing conditions
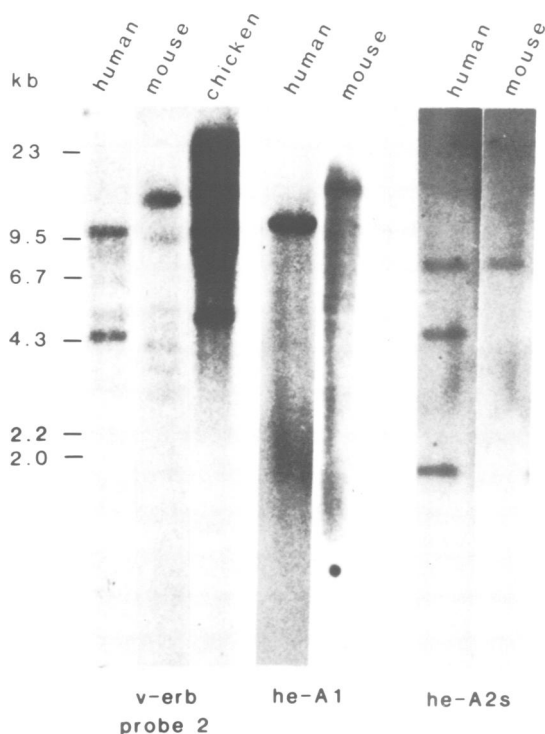
Fig. 4. Analysis of *erb*-related sequences in human and mouse chromosomal DNA. The human DNA was digested with *Eco*RI and the mouse DNA with *Hind*III; the resulting fragments were separated in an agarose gel, transferred to a nitrocellulose filter and hybridized with the probes indicated in the figure. The chicken DNA was digested with *Sac*I. The hybridization with the *v-erbA + B* probe was carried out under moderately stringent conditions, but the probes derived from the human *erbA* clones were used under stringent annealing conditions.
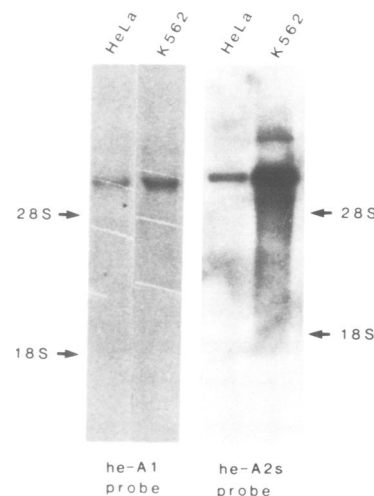

Fig. 5. Analysis of *erbA*-related RNAs in human cells. Polyadenylated RNA was isolated from whole HeLa or K562 cells. The RNA was subjected to electrophoresis in denaturing gels and was transferred to nitrocellulose filters. Hybridizations were carried out with the probes indicated in the figure.

that were used (0.9 M NaCl, 50% formamide at 37°C).

To investigate if additional *erb*-related sequences were present in the human genome, placental DNA was cleaved with restriction enzymes and the resulting fragments were electrophoretically separated and transferred to nitrocellulose filters. When the he-A1 probe was used for hybridization under stringent conditions, a single *Eco*RI fragment of 9.7 kb was detected (Figure 4). A similar experiment with *Hind*III-cleaved mouse DNA detected a 14-kb fragment (Figure 4), indicating that no other genes closely related to the *erbA* gene exist in the human and mouse genomes. The 9.7-kb *Eco*RI fragment is larger than the corresponding fragment in the λhe-A1 clone (Figure 1E), probably attributable to the addition of *Eco*RI linkers to the partial *Hae*III/*Alu*I fragments used for establishing the library. The he-A2s probe reacts with three human *Eco*RI fragments of 2.0, 4.3 and 7 kb in size (Figure 4). Since the probe used in this experiment was derived from a 2-kb *Eco*RI fragment located internally in the λhe-A2 clone, the 2.0-kb fragment detected in the filter hybridization experiment represents its cognate fragment (Figure 1E). However, the annealing to the 4.3- and 7.0-kb fragments indicate the presence of one or two additional *erbA2*-related sequences in the human genome. In the mouse DNA only a 7.0-kb *erbA2*-related *Hind*III fragment was detected (Figure 4), suggesting the presence of only one *erbA2*-related gene in the mouse genome. In an effort to detect additional *erb*-related sequences in the human and mouse genomes, we hybridized *v-erbA + B* probe (probe 2) to fragments of human and mouse chromosomal DNA. Figure

4 shows that two fragments in human DNA, 9.7 and 4.3 kb, hybridize strongly and that these fragments co-migrate with fragments detected with the he-A1 and he-A2s probes described above. In addition, several weakly hybridizing fragments were detected, including the 2.0- and 7.0-kb fragments detected with the he-A2 probe. In mouse DNA the *v-erbA + B* probe detected one strongly hybridizing fragment, which co-migrated with the fragment detected with the he-A1 probe (Figure 4). Several smaller and less strongly hybridizing fragments were detected as well, and they probably represent more distantly related *erbA* and/or *erbB* sequences. The hybridization of the *v-erbA + B* probe to chicken DNA is also shown in Figure 4 where the predominant fragments at 8.1, 7.5 and 5.0 kb can be discerned (see Vennström and Bishop, 1982). A comparison of the intensities of hybridization between the avian and mammalian DNAs suggests that even the most strongly hybridizing mammalian fragments encode *erb* sequences that have diverged significantly from the avian genes, since the experiment was carried out under moderately stringent hybridization conditions.

Transcription of erbA RNA

The expression of the putative human *erbA* genes was investigated by analysing *erb*-related RNAs in the human HeLa and K562 cell lines; the latter has been characterized as a haemopoetic cell line that can be differentiated along the erythroid lineage (Lozzio and Lozzio, 1975; Andersson *et al.*, 1979). Polyadenylated RNA was prepared from whole cells, subjected to electrophoresis in denaturing agarose gels and the RNA was transferred to nitrocellulose filters and hybridized with radioactive probes. Figure 5 shows that the he-A1 probe detects equal amounts of 5.0-kb RNA species in both types of cells. A 5.0-kb RNA species was also detected in both cell lines with the he-A2s probe, but in this case the amount of RNA was considerably higher in the K562 cells (Figure 5). In addition, two less abundant species of RNA were detected in the K562 cells; they might represent nuclear RNA species and/or cytoplasmic RNAs transcribed from other *erbA2* related genes.

Discussion

The data demonstrate that the human and mouse genomes contain two types of genes homologous to the avian *erbA* gene. The two types seem to have diverged significantly from each other as judged by the analysis of the human genes. (i) The *erbA2* gene has homology with only part of the *v-erbA* gene whereas the *erbA1* gene has homology with all regions of *v-erbA* tested. (ii) The two human *erb*-genes we isolated cross-hybridized poorly and efforts to study their relatedness by heteroduplex analysis have failed (B.V., unpublished observations). However, hybridization data suggest that the 3'-terminal parts of the genes have the greatest homology (Figure 2 and unpublished data). (iii) The human *erbA1* and *erbA2* genes we isolated occupy different genetic environments since the clones have distinct restriction maps, and different types of repetitive sequences can be found in both clones at non-reciprocal locations (M.J., unpublished data). On the other hand, the additional *erbA2*-related sequences in the human genome detected by Southern analysis seem to hybridize very strongly to the *erbA2* probe (Figure 4), suggesting that the *erbA2* sequences are closely related.

The human sequence related to the *v-erbB* gene showed only weak homology with parts of the viral *erbB* gene. A detailed study of the properties of the human *erbB* sequence has so far been hampered by the presence of multiple repetitive sequences in its vicinity (M.J., data not shown). DNA sequence analysis is clearly required to establish the relationship between the human and viral *erbB* sequences.

We found one polyadenylated RNA species related to the human *erbA1* gene in the two types of cells tested, and since only a single *erbA1* gene was found in the human genome, the gene is not likely to represent a pseudogene. One major and several minor species of RNA related to the *erbA2* genes were also found, but we cannot at present determine if the minor RNAs are nuclear precursor species and/or if they originate from different *erbA2* genes. Neither can we exclude that some members of the *erbA2* gene family are pseudogenes. That only one species of mRNA is observed from the human *erbA1* gene contrasts with the expression of the avian *erbA* genes: in both chicken and quail cells two different-sized, equally abundant polysomal RNA species can be detected (Vennström and Bishop, 1982; B.V., unpublished data).

The observation that only the *erbA2* and not the *erbA1* transcription is elevated in the K562 cells suggests that the transcription of these genes is differentially regulated, and indicates that the proteins encoded by the two genes may have distinct functions. It is also possible that the elevated *erbA2* gene expression is in some way related to the neoplastic properties of the K562 cell line; this requires further investigation, particularly since it is known that the *erbA* gene is not sufficient for the induction of erythroblastosis in chickens (Frykberg *et al.*, 1983).

The existence of several cellular genes related to retroviral oncogenes is not without precedent. The *v-has* oncogene of Harvey sarcoma virus is homologous to two different rat cellular genes (DeFeo *et al.*, 1981), and other mammals have between one and 10 copies of homologues to *v-has* (Chattopadhyaya *et al.*, 1982; Chang *et al.*, 1982). Similarly, the related oncogene *v-kis* of Kirsten sarcoma virus has one to six cellular homologues depending on the species analyzed *(ibid.)* and some of these genes seem to have diverged significantly from *v-kis* (Chang *et al.*, 1982). Multiple cellular homologues

to viral oncogenes has not only been described for the *has/kis* oncogenes: the human genome contains several divergent sequences related to the *myc* gene as well (Dalla Favera *et al.*, 1982). The occurrence of related but distinct sequences seems to be a hallmark of genes that evolved early in evolution and that have essential functions in cell physiology, as exemplified by the actin, tubulin and globin gene families (Cleveland *et al.*, 1980; Maniatis *et al.*, 1980). The evolutionary conservation of the *erbA* genes thus indicates that they may have a central role in cell physiology.

Materials and methods

Isolation of clones

The human fetal liver DNA library established in Charon 4A by Lawn *et al.* (1978) was screened with probe 2 described below. The plaque hybridizations were carried out under moderately stringent hybridization conditions (see below). Positive plaques were purified until homogeneous and phage DNA was prepared as described previously (Vennström and Bishop, 1982).

Hybridization reagents

Fragments to be used as probes were purified from plasmid subclones after excision with the appropriate restriction enzymes. Probe 1 represents the whole AEV genome subcloned in pBR313. Probe 2 *(verbA + B)* represents a 2.5-kb *Pvu*II fragment encoding ~80% of the *erb* sequences of AEV. Probe 3 is a 0.5-kb *Pst*I fragment located entirely within *erbA*. Probe 4 is a 1.1-kb *AvaI-Bam*HI fragment encoding 3'-terminal parts of *erbA* and 5'-terminal parts of *erbB*; it overlaps probe C by ~0.1 kb. Probe 5 is a 0.5-kb *Bam*HI fragment and probe 6 is a 0.5-kb *Bam*Hi-*Eco*RI fragment; both fragments are located in *erbB*. The locations of the fragments in the AEV genome and their validation have been described in previous communications (Sheiness *et al.*, 1981; Vennström and Bishop, 1982). DNA fragments to be labeled *in vitro* were denatured by boiling and subsequently used as templates for avian myeloblastosis virus reverse transcriptase. Oligomers of calf thymus DNA were used as primers (Hughes *et al.*, 1979).

Analysis of DNA and RNA

Human DNA was prepared from human placenta and mouse and chicken DNAs were prepared from embryos as described previously (Hughes *et al.*, 1979). Agarose gel electrophoresis and transfer to nitrocellulose filters was carried out as described by Southern (1975). Hybridizations of labeled probe to nitrocellulose filters under moderately stringent conditions was carried out by incubation at 37°C in a buffer containing 0.9 M sodium chloride and 50% formamide as described by Shank *et al.* (1978). The buffer used for hybridization under stringent criteria contained 0.45 M sodium chloride and 50% formamide; incubation was at 41°C. The filters were washed repeatedly after hybridization with 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na-citrate), 0.1% SDS at 37°C for moderate stringency and 55°C for high stringency hybridization.

Polyadenylated RNA was purified from whole cells as described previously (Vennström and Bishop, 1982). RNA was subjected to electrophoresis in denaturing agarose gels containing 2.2 M formaldehyde (Vennström *et al.*, 1982). Separated RNA was transferred to nitrocellulose filters as described by Thomas (1980) and hybridized with ³²P-labeled probes as described by Alwine *et al.* (1977).

Acknowledgements

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