Detection of an element of the SV40 late promoter in vectors used for expression studies in COS cells

Roberto Cattaneo*, Hans Will, Gholamreza Darai[1], Eberhard Pfaff and Heinz Schaller

Department of Microbiology, University of Heidelberg, Im Neuenheimer Feld 230, and [1]Institute for Medical Virology, University of Heidelberg, Im Neuenheimer Feld 324, D-6900 Heidelberg, FRG

Plasmids containing hepatitis B virus (HBV) DNA and a 232-bp SV40 DNA fragment encoding the origin of replication were constructed. When introduced by transfection into COS cells, these plasmids directed the synthesis of hepatitis B surface antigen. S1 mapping of the mRNAs covering the S gene showed that transcriptional initiation was promoted by the interaction of HBV sequences with an SV40 promoter element: transcription started on HBV DNA but had several properties of SV40 late transcription. The detection of a promoter element in an SV40 origin fragment commonly used in the COS system is important for the interpretation of data deriving from expression studies in COS cells.
Key words: COS system/gene transfer/SV40 late promoter/ S1 mapping/hepatitis B surface antigen

Introduction

The study of the expression of eucaryotic genes has been greatly facilitated by gene transfer. Initially the transcription of cloned genes was analysed using the co-transformation procedure (for review, see Pellicer *et al.*, 1980) which is based on the introduction of a gene of interest and a selectable marker into eucaryotic cells, whereby multiple copies of both genes eventually integrate into the host genome. This procedure requires several weeks and yields low and variable levels of mRNA. To overcome these limitations, Mellon *et al.* (1981) have described a system allowing the study of transcriptional activities deriving from unintegrated, freely replicating DNA molecules. This system is based on the observation (Myers and Tijan, 1980) that shuttle vectors containing a plasmid replicon and the SV40 origin of replication are efficiently amplified in cells producing SV40 large T antigen (COS cells, Gluzman, 1981). An α-globin gene inserted into such a shuttle vector and introduced into COS cells by transfection was rapidly, efficiently and correctly transcribed and processed (Mellon *et al.*, 1981).

We have analysed the expression of the major protein of hepatitis B virus (HBV) (for reviews, see Tiollais *et al.*, 1981; Will *et al.*, 1982b), the surface antigen (HBsAg), in COS cells. Using this transient assay system we found that 60 h after transfection SV40-HBV hybrids had replicated up to 20 000 molecules per transfected cell, and up to 1.2×10^6 molecules of HBsAg per cell were produced. Initiation of transcription was promoted by the cooperative interaction of SV40 and HBV sequences.

Results

In an attempt to express HBsAg in COS cells, we con-

structed shuttle vectors containing HBV DNA, the SV40 origin of replication and a bacterial plasmid. These SV40-HBV plasmids can replicate both in *Escherichia coli* and COS cells. Plasmid pSH2.1 (Figure 1A) consists of an *E. coli* vector in which a fragment of 232 bp, harboring an intact SV40 origin of replication, and a complete HBV genome, linearized ~150 nucleotides upstream of the S gene, have been inserted. Plasmid pSHH2.1 is identical to pSH2.1, except that a head to tail tandem duplication of the HBV genome was inserted. In plasmid pSH2.6, the S gene was joined to an intact SV40 late promoter (Figure 1B).

Replication of SV40-HBV hybrids in COS cells

To quantify the amplification of the SV40-HBV plasmids in COS cells, total cellular DNA was extracted 60 h after transfection. This DNA was analysed by Southern blotting after cleavage with restriction endonuclease HindIII, which linearizes the plasmids. The results obtained indicated that the vector without the HBV insert (Figure 2, lane pSV08) replicated to ~100 000 copies per transfected cell, whereas 5–50 times fewer copies of plasmids containing HBV sequences were detected (compare lane pSV08 with lanes pSH2.1, pSHH2.1 and pSH2.6 in Figure 2). These data were confirmed by mixed transfection experiments, in which pSV08 always replicated more efficiently than the HBV-containing plasmids; among these pSH2.1 replicated with the highest yields of DNA. Furthermore, DNA extracted from cells transfected with plasmids containing dimers of the HBV genome showed, in addition to the expected DNA fragment,
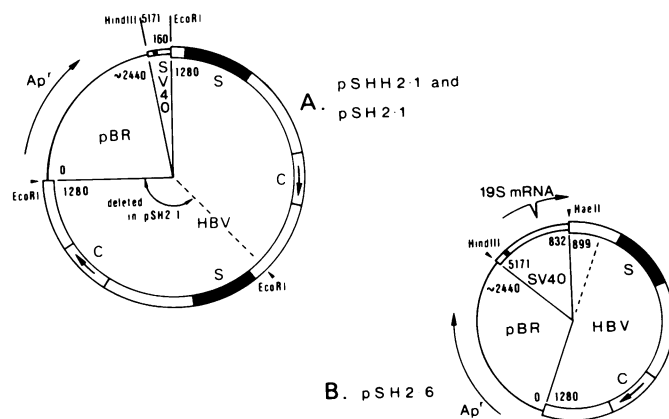

Fig. 1. Plasmids used. (A) Plasmid pSHH2.1 (Will *et al.*, 1982a) is 8.5 kb in length and contains a tandem duplication of a 3.2-kb EcoRI fragment encoding the complete HBV genome (Charnay *et al.*, 1979) inserted into vector pSV08 (Learned *et al.*, 1981). Nucleotides delimiting the HBV, SV40 and pBR sequences are indicated according to the conventions of Pasek *et al.* (1979), Buchman *et al.* (1981) and Sutcliffe (1979), respectively. The HBV S gene (S) and core gene (C) are indicated, as are the pBR penicillinase gene (Ap^r) and the SV40 origin of replication (◼). Plasmid pSH2.1 (Will *et al.*, 1982b) is 5.32 kb in length and contains a single copy of the HBV genome (the HBV genome deleted from pSHH2.1 is indicated). (B) Plasmid pSH2.6 is 6.37 kb in length and was constructed by ligation of a 904-bp HindIII-HaeII fragment of SV40 DNA encoding the complete late promoter and the 19S intron (the 19S mRNA is indicated) with a 5.47-kb HindIII-HaeII fragment of pSHH2.1 encoding 1.12 HBV genomes and the Ap^r gene of pBR322.
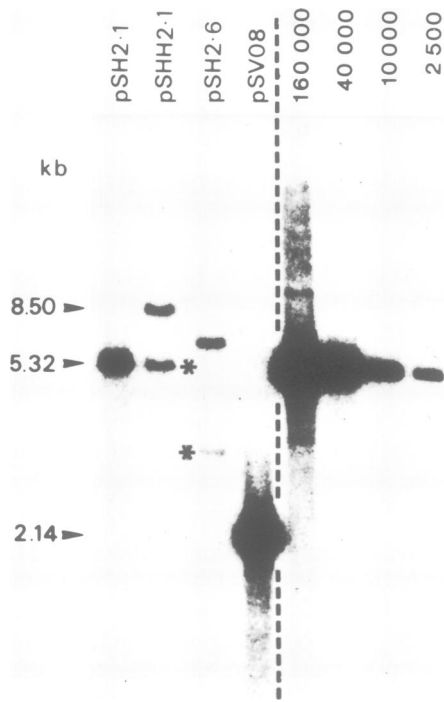
Fig. 2. Replication of SV40-HBV hybrids in COS cells as determined by Southern blotting. In the first four slots, 1 μg of total cellular DNA extracted from COS cells 60 h after transfection using a proteinase K / SDS/phenol procedure (Summers *et al.*, 1975) was restricted with *Hind*III, separated according to size on a 1.2% agarose gel, blotted onto a nitrocellulose filter (Southern, 1975) and hybridized with nick-translated pSV08 (specific activity: ~10⁷ c.p.m./μg DNA). In the four slots on the right, pSH2.1 linearized with *Hind*III was used as hybridisation marker: plasmid DNA corresponding to 160 000, 40 000, 10 000 or 2500 copies of plasmid/cell were loaded. To estimate the number of plasmids per cell we assumed a complexity of 3 x 10⁹ bp for the haploid COS cell genome and a transfection efficiency of 1%. Bands shorter than the original plasmids are indicated with an asterisk in lanes pSHH2.1 and pSH2.6.

an extra band 3.2 kb shorter than the original vector corresponding to 10−50% of total plasmid DNA (asterisk in Figure 2, lanes pSHH2.1 and pSH2.6). This suggests that one copy of the HBV genome was lost, probably by intramolecular homologous recombination. Since these extra bands were not seen in the plasmid preparation used for transfection, it is conceivable that recombination had occurred in COS cells. A less likely interpretation would be that a minor contamination of the plasmid preparations used for transfection is preferentially amplified in COS cells.

Transcripts mapping in the S region

To investigate whether the S genes encoded in the three SV40-HBV plasmids used were transcribed in COS cells, we analysed the RNAs, extracted 48 h after transfection, by S1 mapping. We first used a probe covering 1000 bp of HBV DNA upstream of the S gene and 360 bp of its coding region (probe A, Figure 3). RNA extracted from COS cells transfected with pSH2.1 and pSHH2.1 protected 235 or ~500 nucleotides of probe A (Figure 4A, lanes pSH2.1 and pSHH2.1) indicating the existence of two RNA species. The S1-protected fragment 235 bp in length, corresponds to an RNA species occurring in all three HBV-containing plasmids (Figure 4A, lanes pSH2.1, pSHH2.1 and pSH2.6). Its 5′ end maps in the S gene (Figure 5), within the sequence 1549 TCTCAATTTTCTAG/GGG 1567. RNA 5′ ends detected by the S1 technique can correspond to initiation sites of
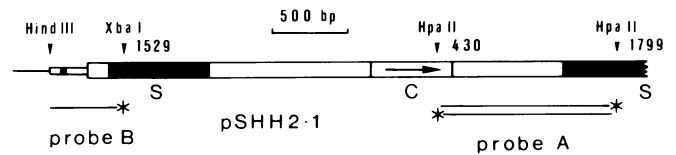

Fig. 3. Probes used for S1 analysis of the transcripts produced in COS cells. Symbols for SV40, HBV and plasmid DNA are as in Figure 1. Probe A was a 1369-bp *Hpa*II fragment labelled at both 5′ ends and was derived from the second S gene of pSHH2.1. Probe B was a 481-bp *Xba*I-*Hind*III fragment labelled at the *Xba*I 5′ end and was derived from the SV40-HBV junction of pSHH2.1.
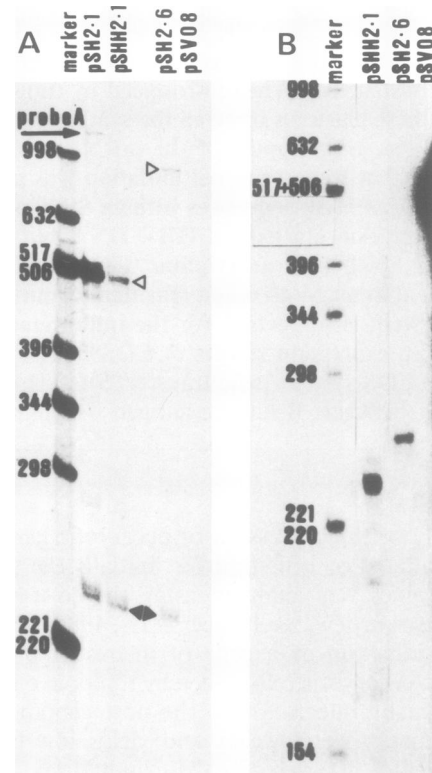

Fig. 4. A and **B:** S1 analysis of the transcripts produced in COS cells. Cytoplasmic RNA from ~5 x 10⁶ cells extracted 48 h after transfection according to Dunn *et al.* (1979) was hybridised at 46°C with 0.02 pmol probe A (**gel A**) or at 50°C with 0.02 pmol probe B (**gel B**) according to Weaver and Weissman (1979). The specific activity of the probe was ~5 x 10⁵ c.p.m./μg DNA. Hybrids were analysed on 6% acrylamide sequencing gels dried to enhance sensitivity (Garoff and Ansorge, 1981). On **gel A** the band corresponding to the renatured probe A is indicated with an arrow in lane pSH2.1. On **gel B** the band corresponding to probe B was overexposed and is visible in lane pSV08. RNA extracted from COS cells transfected with pSV08 did not protect either probe A or probe B (**gels A** and **B**, lanes pSV08), as expected for these two negative controls. RNA extracted from pSH2.6 transfected cells protected probe B for ~250 nucleotides (**gel B**, lane pSH2.6), i.e., to the limit of homology of probe B with pSH2.6 DNA. The results of the other hybridisations are discussed in the text.

transcription, to splice acceptor sites or they can derive from destabilisation of the RNA-DNA hybrids resulting from AT-rich fragments. No TATA box is present upstream of the RNA 5′ end, but a short AT-rich region (underlined) and a putative splice acceptor signal ([Py]ₙ NPyAG/G; Mount, 1982) are encoded in this region.

The second protected fragment, 500 bp in length is present in pSHH2.1 and pSH2.1 but not in pSH2.6 transcripts
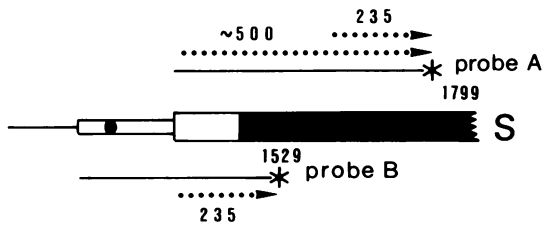
Fig. 5. RNA species produced by plasmids pSH2.1 and pSHH2.1. Symbols for SV40, HBV and plasmid DNA are as in Figure 1. Probe A was drawn only to the limit of co-linearity with the plasmids (the SV40-HBV junction). The RNA species protected by probes A and B are depicted as dotted lines. For details see text.

Fig. 6. Kinetics of pSH2.1 amplification ($\times$), S gene transcription ($\bigcirc$) and HBsAg expression ($+$) as monitored by Southern blotting of the supercoiled plasmids, S1 mapping and enzyme-linked immunoasay of whole cell extracts.

(Figure 4A, lanes pSH2.1, pSHH2.1 and pSH2.6). This observation, in conjunction with its size, indicates that the corresponding RNA starts from a signal close to the SV40-HBV junction, which in these two plasmids is located ~150 nucleotides upstream of the S gene. No RNA species exceeding 500 nucleotides, nor one completely protecting probe A was detected in pSHH2.1 transcripts (Figure 4A, lane pSHH2.1) indicating the absence of an mRNA species covering additional HBV sequences upstream of the S gene.

To map precisely the 5' end of the RNA species initiating close to the SV40-HBV junction in pSH2.1 and pSHH2.1, a probe covering this region (probe B, Figure 3) was used to protect RNA extracted from COS cells transfected with pSHH2.1. This resulted in a strong double band of ~235 nucleotides in length and additional minor bands of 150−250 nucleotides (Figure 4B, lane pSHH2.1). Thus, the major species of pSHH2.1 transcripts have 5' ends situated in the HBV DNA 15 nucleotides downstream of the SV40-HBV junction (Figure 5). Since in this region no splice acceptor signal or AT-rich region is encoded, the most likely interpretation is that these 5' ends correspond to initiation sites of mRNA.

No deviation from the expected results was obtained with the control plasmid pSH2.6. This plasmid contains an S gene joined to an SV40 DNA fragment 904 bp in length encoding the origin of replication, the SV40 late promoter and the 19S intron (Figure 1B). RNA extracted from COS cells transfected with pSH2.6 protected a fragment of probe A of ~900 nucleotides in S1 experiments (Figure 4A, lane pSH2.6). This corresponds to an RNA 5' end at or upstream of the HBV-SV40 junction, which is the limit of sequence homology between mRNA and probe A. Subsequent experiments, using a probe covering the SV40-HBV junction, showed that transcription was initiated further upstream on SV40 sequences. With this probe, RNA 5' ends were mapped around SV40 nucleotide 550 (data not shown), corresponding to the SV40 19S RNA splice acceptor site. Thus, we conclude that, in plasmid pSH2.6, transcription of the S gene is initiated by the SV40 late promoter.

HBsAg expression mechanism

To determine whether S gene transcription was followed by expression, whole cell extracts of COS cells harvested 60 h after transfection were tested for the presence of HBsAg by an enzyme-linked immunoassay (HBsAg ELISA, Behringwerke). A positive signal 5−10 times above background was obtained with cells transfected with plasmid pSH2.1, cells transfected with plasmid pSV08 or mock-transfected were negative. By calibrating the test with a standard of known concentration, we established that this corresponds to
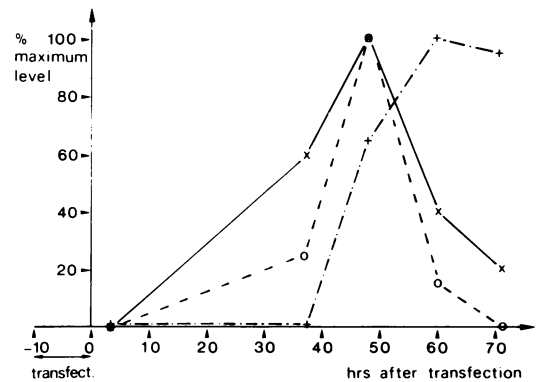
~0.25 ng HBsAg per 10^6 cells. Approximately the same quantity of antigen was detected in the medium. This corresponds to a production of ~1.2 x 10^6 molecules HBsAg per transfected cell, assuming a transfection efficiency of 1% (Materials and methods) and a mol. wt. of 25 000 for HBsAg. Lower levels (2−5 times above background in ELISA) of HBsAg were detected in COS cells transfected with plasmid pSHH2.1 or pSH2.6, which correlates with the less efficient replication of these plasmids (Figure 2).

To gain insight into the mechanism of HBsAg expression, we measured the steady-state levels of supercoiled plasmid DNA, S gene transcripts and HBsAg present in COS cells at different times after transfection. In COS cells transfected with pSH2.1, DNA replication, as monitored by the presence of supercoiled plasmid DNA, and S gene transcription correlated, reaching their maximum 48 h after transfection and then dropping (Figure 6), whereas HBsAg continued to accumulate at later times, probably because of its greater stability. The quantity of total plasmid DNA, as monitored by Southern blotting of restricted cellular DNA, remained constant at its maximum level 48 and 60 h after transfection, rapidly declining after 72 h. Expression of hepatitis B e or core antigen could not be detected by radioimmunoassay (Abbot-HBe™).

Discussion

For the analysis of the expression of cloned genes, the COS system has a distinct advantage over the stable co-transformation procedure, in that plasmids of defined structure can replicate to a high copy number, thus facilitating and accelerating the transcriptional and translational analysis. However, our study of the expression of HBsAg in this system has encountered two unexpected difficulties: HBV-SV40 hybrids were not amplified as well as plasmids containing only SV40 sequences and transcriptional interferences of vector sequences were noticed. The reason for the low amplification of the HBV DNA-containing plasmids is not entirely clear: in our study low amplification correlates with increasing size of the plasmids (Figure 2), but in a previous report replication of vectors containing the SV40 origin and α-globin sequences was not size dependent (Mellon *et al.*, 1981). It is also conceivable that the HBV genome contains sequences poisonous for COS cells (Lusky and Botchan, 1981).

The second difficulty encountered is related to the detection of an active promoter element in an SV40 origin fragment commonly used in COS system and initially assumed to be free from the interference of highly active viral promoters (Mellon et al., 1981). We have observed that the transcription of the S gene in pSH2.1 and pSHH2.1 shares two properties with SV40 late transcription: multiple RNA 5' ends are produced (Figure 4B) and transcription correlates directly with DNA replication (Figure 6). Moreover, the promoter active in pSHH2.1 has a similar strength to the SV40 late promoter active in pSH2.6 (Figure 4B), both plasmids replicating to about the same level (Figure 2). Thus, we conclude that an SV40 late promoter element activates initiation of transcription around HBV nucleotide 1295 in plasmids pSH2.1 and pSHH2.1. These HBV sequences are transcriptionally inactive when flanked by a longer stretch of HBV DNA, as in plasmid pSH2.6 (Figure 4A, lane pSH2.6).

In more recent experiments (R.Cattaneo, unpublished results), the nature of this SV40 promoter element has been studied by using analogous SV40-HBV plasmids in which the size of the SV40 origin fragment has been reduced from 232 bp to its minimal functional length (85 bp, Learned et al., 1981). Even with these vectors, transcripts deriving from the SV40 late promoter were identified, thus indicating that at least part of the SV40 late promoter cannot be dissociated from the SV40 origin of replication. A similar conclusion has recently been drawn from the transcriptional analysis of SV40 mutants, which suggested a direct role for the origin of DNA replication in the activation of late transcription (Contreras et al., 1982).

The sites of transcription initiation deriving from the SV40-HBV promoter had shifted 50 − 150 nucleotides towards the SV40 origin in comparison with the initiation sites of the late transcripts of SV40 virus (Ghosh et al., 1978). A similar shift had been observed in SV40 mutants (Piatak et al., 1981) and in a SV40-preproinsulin hybrid (Gruss and Khoury, 1981). Thus, we conclude that the position of the start sites deriving from the SV40 late promoter are determined by the interaction of the promoter element at the origin of replication with its neighbouring sequences.

Recently, Pourcel et al. (1982) have investigated the transcriptional activity of HBV DNA after its integration in the genome of mouse L cells and have localized the site of initiation of an RNA covering the S gene in a region situated 500 −900 nucleotides upstream of it. In monkey cells, and with the freely replicating SV40-HBV plasmid pSHH2.1, a similarly active promoter could not be detected, the limits of detection being <10% of the signal produced by the SV40-HBV 'late' promoter. The reason for the discrepancy is not clear: several differences between the two systems including cell specificity, mode of replication and technique of transfection could account for it. The further possibility that the cloned DNA used by us to transfect COS cells might be defective in promoter function can be ruled out because the same DNA has been shown to yield infectious virus in chimpanzees (Will et al., 1982a, 1982b).

Materials and methods

Growth and transfection of COS cells; transfection efficiency

COS7 cells (Gluzman, 1981) were grown at 37°C in Basal Medium Eagle with Earle's salt (BME-Flow Laboratories) supplemented with 10% newborn calf serum (NCS) and passaged at 1:5 dilutions. Transfections were performed using a modified DEAE-dextran procedure (Sompayrac and Danna, 1981): subconfluent COS cells in a 80 cm² Falcon flask were washed twice

with BME and incubated for 8 − 12 h in 2.5 ml of transfection cocktail (1 x BME, 50 mM Tris pH 7.3, 200 μg/ml DEAE-dextran, mol. wt. 2 000 000) in which 1 pmol of plasmid DNA had been dissolved. Cells were washed twice and incubated at 37°C or trypsinized, divided into aliquots and incubated at 37°C in 1 x BME, 10% NCS for various periods of time as indicated.

Transfection efficiency was determined by comparing the quantity of SV40 DNA produced by COS cells 60 h after the end of transfection or after infection with wild-type SV40 DNA or virus. Since virus-infected cells produced ∼100 times more than DNA-transfected cells (as monitored by Southern blotting) we estimate that ∼1% of the COS cells were transfected.

References

Buchman,A.R., Burnett,L. and Berg,P. (1981) in Tooze,J. (ed.), DNA Tumor Viruses, Cold Spring Harbor Laboratory Press, NY, pp. 799-841.

Charnay,P., Pourcel,C., Louise,A., Fritsch,A. and Tiollais,P. (1979) Proc. Natl. Acad. Sci. USA, 76, 2222-2226.

Contreras,R., Gheysen,D., Knowland,J., van de Voorde,A. and Fiers,W. (1982) Nature, 300, 500-505.

Dunn,A.R., Mathews,M.B., Chow,L.T., Sambrook,J. and Keller,W. (1978) Cell, 15, 511-526.

Garoff,H. and Ansorge,W. (1981) Anal. Biochem., 115, 450-457.

Ghosh,P.K., Reddy,V.B., Swinscoe,J., Lebowitz,P. and Weissman,S.M. (1978) J. Mol. Biol., 126, 813-846.

Gluzman,Y. (1981) Cell, 23, 175-182.

Gruss,P. and Khoury,G. (1981) Proc. Natl. Acad. Sci. USA, 78, 133-137.

Learned,R.M., Myers,R.M. and Tijan,R. (1981) in Ray,D.S. (ed.), The Initiation of DNA Replication, Academic Press, NY, pp. 555-566.

Lusky,M. and Botchan,M. (1981) Nature, 293, 79-81.

Mellon,P., Parker,V., Gluzman,Y. and Maniatis,T. (1981) Cell, 27, 279-288.

Mount,S.M. (1982) Nucleic Acids Res., 10, 459-472.

Myers,R.M. and Tijan,R. (1980) Proc. Natl. Acad. Sci. USA, 77, 6491-6495.

Pasek,M., Goto,T., Gilbert,W., Zink,B., Schaller,H., MacKay,P., Leadbetter,G. and Murray,K. (1979) Nature, 282, 575-579.

Pellicer,A., Robins,D., Wold,B., Sweet,R., Jackson,J., Lowy,I., Roberts, J.M., Sim,G.-K., Silverstein,S. and Axel,R. (1980) Science (Wash.), 209, 1414-1421.

Piatak,M., Subramanian,K.N., Roy,P. and Weissman,S.M. (1981) J. Mol. Biol., 153, 589-618.

Pourcel,C., Louise,A., Gervais,M., Chenciner,N., Dubois,M.-F. and Tiollais,P. (1982) J. Virol., 42, 100-105.

Sompayrac,L.M. and Danna,K.J. (1981) Proc. Natl. Acad. Sci. USA, 78, 7575-7578.

Southern,E.M. (1975) J. Mol. Biol., 98, 503-517.

Summers,J., O'Connell,A. and Millman,I. (1975) Proc. Natl. Acad. Sci. USA, 72, 4597-4601.

Sutcliffe,G. (1979) Cold Spring Harbor Symp. Quant. Biol., 43, 77-90.

Tiollais,P., Charnay,P. and Vyas,G.N. (1981) Science (Wash.), 213, 406-411.

Weaver,R.F. and Weissman,C. (1979) Nucleic Acids Res., 7, 1175-1193.

Will,H., Cattaneo,R., Koch,H.G., Darai,G., Schaller,H., Schellekens,H., van Eerd,P.M.C.A. and Deinhardt,F. (1982a) Nature, 299, 740-742.

Will,H., Kuhn,C., Cattaneo,R. and Schaller,H. (1982b) in Miwa,M. et al. (eds.), Japan Scientific Society Press, Tokyo pp. 237-247.