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The Cytomegalovirus Enhancer: a Pan-Active Control Element in Transgenic Mice

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In an effort to identify widely active positive regulatory elements, we have examined the action of the cytomegalovirus enhancer-promoter in transgenic mice. These elements activated expression in 24 of 28 tissues tested. The greatest expression was observed in the heart, kidney, brain, and testis. Maximum expression further localized to specific cells within the heart and kidney.

We define a pan-active promoter or enhancer as one that targets expression of a gene to the broadest possible array of tissue types. Gene constructions which contain such pan-active elements are useful for studies designed to assess gene function in a wide range of cell types as well as in certain instances of somatic gene therapy. Indeed, a variety of combinations of promoter and enhancer sequences have been assessed for this purpose in transgenic mice (2, 7, 11, 15).

To identify a broadly expressed and potent promoter-enhancer, the enhancer for the immediate-early (IE) genes of the human cytomegalovirus (CMV) was examined. CMV is infectious in a wide range of tissues (16), and its targeting to cells seems independent of specific virus-receptor interactions (1). Most importantly, in in vitro studies, the CMV enhancer appears to be the most potent tested to date (3).

Our experiments were designed to assess independently the activities of the CMV promoter-enhancer and the CMV enhancer used in conjunction with a heterologous promoter. Two recombinant DNA plasmids were developed to test the role of the CMV enhancer as a tissue-specific regulatory element in transgenic mice. pCNH contains the CMV enhancer in combination with its own promoter directing transcription of the neomycin (G418) phosphoryltransferase gene in one transcription orientation (Fig. 1, top). By definition, an enhancer functions independently of orientation; therefore, the heterologous herpes simplex virus thymidine kinase (HSV-tk) promoter, directing transcription of the hygromycin phosphoryltransferase, was placed in the opposite orientation to serve as a reporter gene to assess the activity of the CMV enhancer. The second control recombinant (pTKH) (Fig. 1, bottom) was constructed to contain an enhancerless HSV-tk promoter directing transcription of the hygromycin phosphoryltransferase gene for comparison with the activation of this heterologous promoter by the CMV enhancer.

pTKH (Fig. 1, bottom) is the starting plasmid for the recombinant plasmids used in these experiments. It contains the HSV-tk promoter and termination elements directing expression of the hygromycin resistance gene encoding hygromycin phosphoryltransferase. It was generated from the plasmid pHEBo (13) which contains the HSV-tk promoter and termination sequences from the *Pvu*II site upstream of transcriptional initiation to the *Bst*EII site downstream of the HSV-tk termination sequences. This fragment was subcloned from pHEBo into the *Sph*I site of the Bluescribe (Stratagene, Inc.) vector. An injection fragment used to make transgenic mice (8) was generated from pTKH by cutting at *Sal*I and *Hin*dIII sites.

pCNH (Fig. 1, top) is the test plasmid containing the CMV promoter-enhancer used in these experiments. A plasmid containing the CMV enhancer directing expression of the gene for G418 resistance and the adenosine deaminase gene, pCMV/neo/ada, was obtained from S. H. Orkin (unpublished observations). The HSV-tk–hygromycin sequences from pTKH were excised as an *Xba*I-*Hin*dIII fragment and were cloned into the *Spe*I-*Hin*dIII site of pCMV/neo/ada. This replaced the adenosine deaminase sequences of the starting plasmid. It generated a plasmid with the CMV enhancer and promoter directing expression of the neomycin resistance gene in one orientation and CMV-enhanced expression of hygromycin resistance from the HSV-tk promoter in the opposite orientation. To generate an injection fragment, vector sequences were removed by cutting at the *Xmn*I and *Hin*dIII sites of pCNH.

In two independent transgenic lines, the CMV promoter-enhancer sequences directed abundant widespread expression of the reporter transgenes (Fig. 2; Table 1). Three additional lines of transgenic mice carried inactive copies of the transgene. The variation in expression between our CMV transgenic strains is similar to variations observed in other transgenic experiments (8).

For the transgenic mice carrying pCNH plasmid sequences, the combination of the CMV enhancer and promoter directed transcription of the neomycin resistance gene linked to simian virus 40 (SV40) polyadenylation sequences. RNA from these mice was extracted by the method of Chirgwin et al. (4) from the indicated tissues and organs (Fig. 2). These RNAs were analyzed in an RNase protection analysis (6) (Fig. 2A). The antisense RNA probe (SV40 polyadenylation sequence probe) contained RNA corresponding to sequences between the *Nru*I site in the neomycin resistance gene and the *Eco*RI site 3′ to the polyadenylation signal of the plasmid pCNH (8). It protects a fragment

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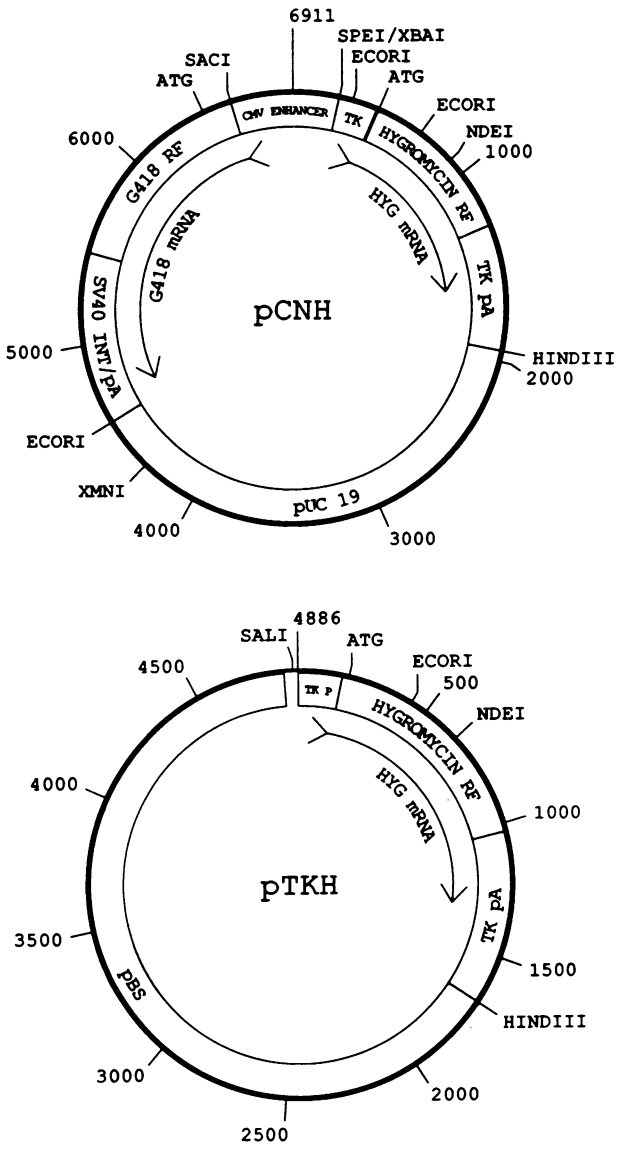

FIG. 1. Construction of fusion genes. pTKH is the starting plasmid for these experiments. It contains the HSV-tk promoter and termination elements directing expression of the hygromycin resistance gene encoding hygromycin phosphoryltransferase. TK P, HSV-tk promoter sequences; RF, reading frame; TK pA, HSV-tk polyadenylation signal and termination sequences; pBS, Bluescribe vector sequences; HYG mRNA, the expected mRNA fusion sequences using the HSV-tk promoter and termination sequences to express hygromycin resistance. pCNH is the test plasmid containing the CMV promoter-enhancer used in these experiments. CMV ENHANCER, the regulatory sequences of the IE region of CMV between the *Spe*I site 5′ to the enhancer and the *Sac*I site at the site of transcriptional initiation (3); TK, HSV-tk promoter; HYGROMYCIN RF, hygromycin resistance-coding sequences; TK pA, HSV-tk termination sequences; pUC 19, vector sequences of pUC 19; SV40 INT/pA, the intron and polyadenylation sequences of SV40; G418 RF, coding sequences for neomycin-phosphoryltransferase/G418 resistance; HYG mRNA, the expected mRNA fusion sequences using the HSV-tk promoter and termination sequences to express hygromycin phosphoryltransferase; G418 mRNA, the expected mRNA fusion sequences using the CMV promoter and SV40 polyadenylation sequences to express neomycin phosphoryltransferase.

810 nucleotides in length between the poly(A) of the mRNA and the splice acceptor site of the SV40 intron.

Transcripts were detected in 24 of 28 tissues tested in the transgenic line which gave the highest levels of expression (TG.EV) using an RNase protection analysis (Fig. 2A). A hierarchy of expression was seen, with the highest levels of transcripts found in the heart, renal medulla, and testis. The next highest levels were found throughout the brain, eye, small intestine, and renal cortex, with lower levels found in the gastrointestinal tract, including the esophagus, stomach, and large intestine. Low to moderate levels of transcription were found in the salivary gland, epididymis, breast, ovary, uterus, lymph node, spleen, thymus, and skin. The potency of the CMV enhancer is reflected in a comparison between its expression in cardiac cells (Fig. 2A, lane HT) and G418-selected cells (Fig. 2A, lane cell) used as a control. Equivalent signals in these lines suggest that the CMV enhancer can naturally direct transgenic expression in heart cells at functional levels comparable to those levels seen in specifically selected cells in tissue culture.

The same spectrum of activity was conveyed to the adjacent HSV-tk promoter by the addition of the CMV enhancer (Fig. 2B). In this case, a hygromycin resistance reporter gene was transcribed from the heterologous HSV-tk promoter in the direction opposite to that of the CMV promoter (Fig. 1, top). The hygromycin–HSV-tk fusion transcript was detected in an RNase protection assay (Fig. 2, bottom) and compared with those in a control transgenic mouse (TG.EI) carrying the HSV-tk–hygromycin fusion gene alone (pTKH; Fig. 1, bottom). The antisense RNA probe contained sequences between the *Nde*I site of the hygromycin resistance gene and the *Hin*dIII site of the plasmid pTKH (Fig. 1). It protects a fragment 700 nucleotides in length corresponding to sequences between the *Nde*I site of hygromycin resistance and the polyadenylation signal of the HSV-tk termination region. It also protects a specific fragment 1,050 nucleotides in length corresponding to full-length transcription from the *Nde*I site through the polyadenylation signal, extending to the *Hin*dIII site.

Transgenic mice bearing the CMV-activated HSV-tk promoter exhibited high levels of transcription in the same tissues as those exhibiting transcripts derived from the CMV promoter-enhancer (compare lanes EV in Fig. 2B with Fig. 2A). Transcription in these organs was activated by the CMV enhancer, because the corresponding organs in the control mice lacking the CMV enhancer were negative for expression. The heart, kidney, eye, small intestine, spleen, thymus, and all portions of the brain contained transcripts at levels that paralleled expression of neomycin-SV40-specific sequences.

That the pattern of expression seen in the CMV promoter-enhancer-bearing line (TG.EV) was due to the regulatory sequences incorporated into the fusion transgene, rather than due to a consequence of a fortuitous insertion site, was supported by studies of a second independently derived transgene-bearing CMV promoter-enhancer fusion gene (TG.ER). Although this second line displayed apparently twofold-lower levels of transgene expression, the tissues in which transcripts were found corresponded to those observed in the first line (TG.EV), including those from the testis, heart, kidney, small intestine, and all areas of the brain (Table 1).

To determine whether variations in expression between tissues were caused by localized expression in specific cells, we determined the spatial expression pattern of the trans-

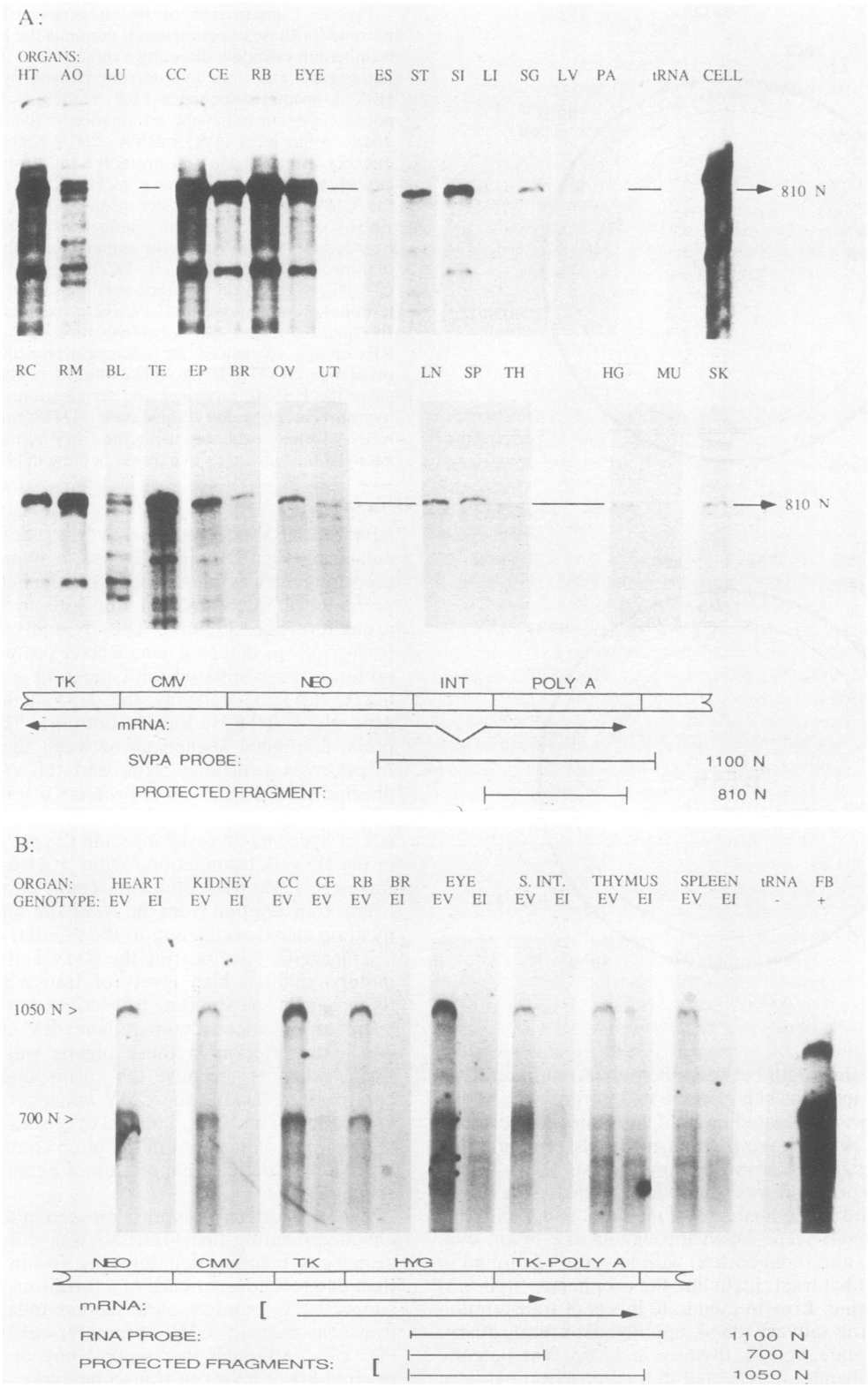

FIG. 2. Expression of transcripts directed by the CMV enhancer and promoter in transgenic mice (TG.EV). (A) Expression directed by the combined CMV promoter-enhancer in the highest-expressing line of pCNH transgenic mice (TG.EV). Total RNA (10 μg) from the indicated organs was used in the RNase protection assay after hybridization to the SV40 polyadenylation sequence probe. The resulting autoradiograms were exposed overnight. HT, heart; AO, aorta; LU, lung; CC, cerebral cortex; CE, cerebellum; RB, remaining brain; EYE, eye; ES, esophagus; ST, stomach; SI, small intestine; LI, large intestine; SG, salivary gland; LV, liver; PA, pancreas; RC, renal cortex; RM, renal medulla; BL, bladder; TE, testis; EP, epididymis; BR, breast (female); OV, ovary; UT, uterus; LN, lymph node; SP, spleen; TH, thymus; HG, Harderian gland; MU, muscle; SK, skin; tRNA, 20 μg of yeast tRNA as a negative control; CELL, 5 μg of total RNA from BALB/c 3T3 cells transfected with pCNH grown in 800 μg of G418 per ml as a positive control; TK, HSV-tk promoter; CMV, CMV enhancer

TABLE 1. Transcription in organs of transgenic lines

Organ-specific RNA from:	Enhancer-promoter-directed expression[a] *in transgenic line:*		
	TG.EV (CMV)	TG.ER (CMV)	TG.EI (HSV-tk)
Circulatory			
Heart	++++	++	−
Aorta	+++	+	
Lung	−	−	−
Neural			
Brain (whole)	+++	++	−
Cerebral cortex	+++	++	
Cerebellum	+++	+	
Remaining brain	+++	++	
Eye	+++	+++	−
Gastrointestinal			
Esophagus	+	−	
Stomach	++	+	
Small intestine	++	+	−
Large intestine	+	−	−
Salivary gland	+	−	−
Liver	−	−	−
Pancreas	−	−	
Genitourinary			
Kidney	+++	++	−
Cortex	++	++	
Medulla and papilla	+++	+++	
Bladder	++	+	
Testis	+++	++	
Epididymis	++		
Breast	+		
Ovary	+	−	−
Uterus	+	−	−
Lymphoid			
Lymph node	+		
Spleen	+	+	−
Thymus	+	−	−
Other			
Harderian gland	+		
Muscle	−	−	
Skin	+	−	

[a] −, No expression; +, relatively low level of expression; ++, moderate level of expression; +++, high level of expression; ++++, highest level of expression.

gene by using in situ hybridization analysis (13) of kidneys and hearts from the highest-expressing line (TG.EV). Transgene expression in the kidneys was highest in the cells forming the renal papillae of the renal medulla (Fig. 3A). In the renal cortex, expression was highest in the epithelial cells lining collecting tubules (Fig. 3B). Thus, the CMV transgene is expressed at high levels in specific cell types of both the renal cortex and the medulla but is also expressed at lower levels throughout the kidney.

Expression of the CMV transgene was similarly analyzed in hearts from day-old transgenic mice. Strong hybridization was seen throughout the ventricles and the atria (Fig. 3C). Clusters of cells expressing the transgene at the highest levels were scattered throughout both ventricles. In contrast, no hybridization signal was detected in the cells forming the cardiac valves (Fig. 3C, compare panels 2 and 3).

Since the CMV infects a broad variety of tissues and encodes a powerful enhancer (3, 14), it might be expected to direct widespread expression in a transgenic mouse system. Our results indicate that it is indeed expressed in a substantial number of tissues in these animals (Table 1). However, the variation in transgene expression between organs and cell types may also be informative regarding the nature of CMV infection.

The CMV enhancer plays a crucial role in the life cycle of the CMV. The tissue specificities of many viruses depend on unique interactions between viruses and particular cell surface receptors. In contrast, CMV is thought to interact with β_2-microglobulin, a virtually ubiquitous cell surface protein, via a viral gene homologous to class I histocompatibility antigens (1). After nonspecific entry into cells, the initial expression of viral genes is directed from the CMV IE gene promoter-enhancer. Transcription of the IE genes is then required to regulate expression of subsequent stages in the life cycle of the virus (9, 12); therefore, tissue-specific host factors that interact with the CMV IE enhancer are likely to be essential for replication of the virus. The broad, but nonuniform, activity of the CMV enhancer observed in our transgenic mice may explain why only certain tissues are infected by the virus.

In addition, the IE gene products can both activate and repress expression from the CMV IE enhancer (9, 12). In the initial stages of infection, they activate their own expression and expression of other viral genes as the virus usurps the transcriptional machinery of the host (12). Subsequently, products of IE genes can either directly or indirectly downregulate their own expression in an autoregulatory cycle (9). Therefore, it may be significant that high levels of activity of the enhancer in the hearts and kidneys of transgenic mice correspond to organs in which CMV produces latent infections (5). Moderate levels of activity correspond to organs in which lytic infections are observed, including the salivary gland, breast, reproductive tract, and gastrointestinal tract (16). Thus, the CMV enhancer may represent a first regulatory step which participates in the choice between latent and productive infections.

While the extent of activity of the CMV promoter-enhancer may have implications for the pathophysiology of CMV infection, its broad activity suggests that it might be useful as a regulatory element in somatic gene therapy. For example, the CMV promoter offers the option of using

and promoter; NEO, coding sequences for G418 resistance; INT, splice donor and acceptor sequences from the small T antigen of SV40; POLY A, polyadenylation and termination sequences from the large T antigen of SV40. (B) Expression of transcripts in the presence of the CMV enhancer acting on the HSV-tk promoter. Total RNA (20 μg) extracted from the indicated organs and tissues of mice from this line was used to compare CMV-enhanced HSV-tk-promoted transcripts (TG.EV) with a second line containing HSV-tk-promoted transcripts lacking an enhancer (TG.EI). RNA PROBE, Antisense RNA used in the RNA protection analysis. Yeast tRNA (20 μg) was included as a negative control (tRNA −), and total RNA (5 μg) from BALB/c 3T3 cells transfected with the plasmid pCNH grown in 100 μg of hygromycin was included as a positive control (fibroblast [FB +]). NEO, neomycin resistance-coding sequences; CMV, CMV enhancer; HYG, hygromycin resistance-coding sequences; TK-POLY A, HSV-tk termination and polyadenylation sequences. The rest of the abbreviations are as defined for panel A.

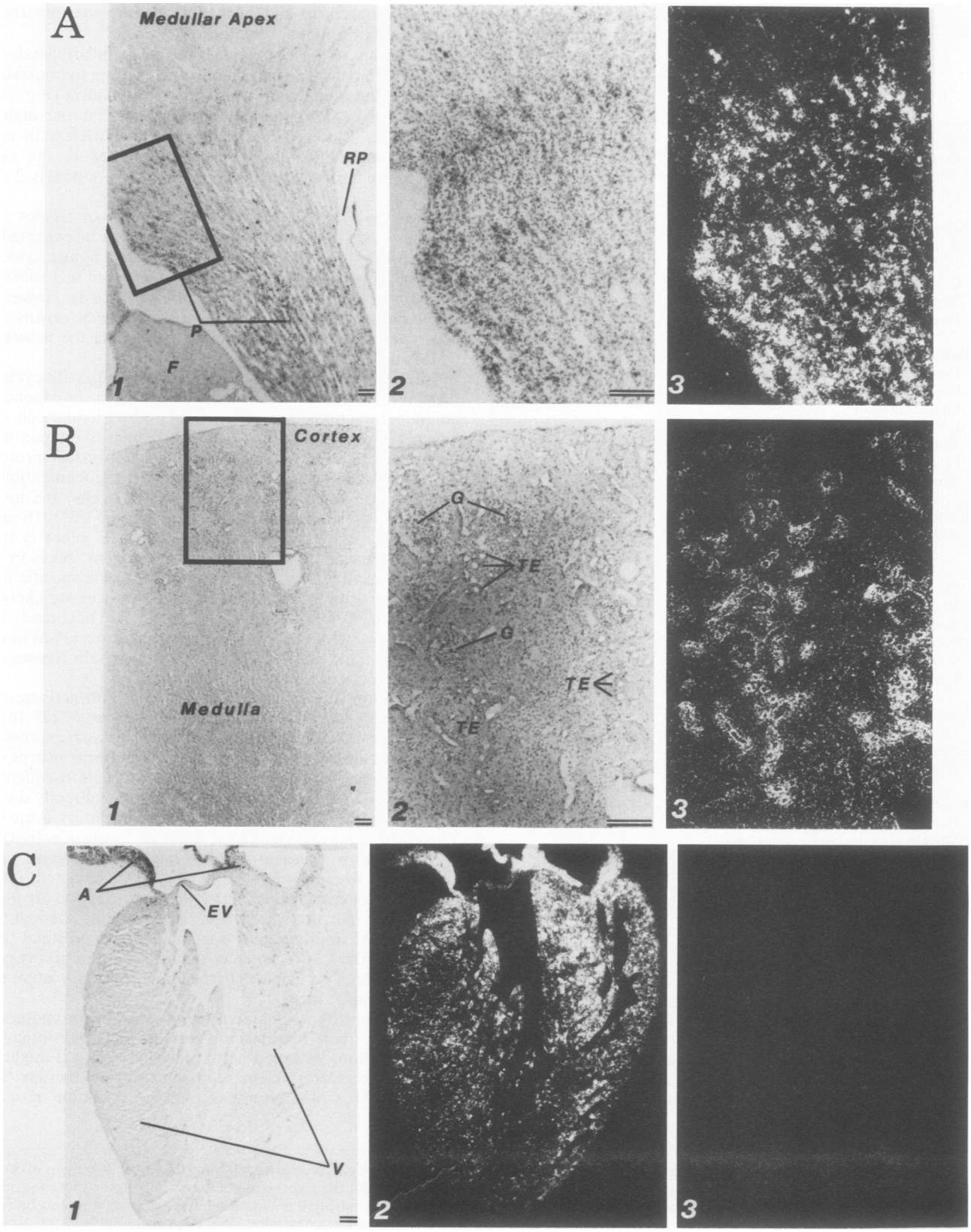

FIG. 3. In situ analysis of the expression pattern of the CMV transgene in kidneys and hearts. In situ mRNA hybridization was carried out by using our previously published technique (17) with [^{35}S]UTP-labeled antisense riboprobes corresponding to the SV40 poly(A) region (Fig. 2A). The spatial pattern of expression of the CMV transgene was analyzed in adult kidneys and 1-day-old neonatal hearts from TG.EV animals. (A) Expression pattern in cells of the medullar apex of the kidney. 1, Bright-field illumination showing the renal papilla (P) and renal

pelvis (RP) of a TG.EV kidney. F, Fat tissue. Box indicates enlargements shown in panels 2 and 3. 2, Enlargement of the renal papilla. 3, Dark-field illumination of panel A2. (B) Expression pattern in cells of the renal cortex. 1, Bright-field illumination showing renal cortex and medulla. Box indicates enlargement shown in panels B2 and B3. 2, Enlargement of renal cortex as shown in panel B1. G, Glomerulus; TE, tubular epithelium. 3, Dark-field illumination of panel B2. (C) Expression pattern in a 1-day-old neonatal heart. 1, Bright-field illumination of a longitudinal section of a TG.EV transgenic neonatal heart. A, Atria; EV, endocardial valve; V, ventricle. 2, Dark-field illumination of panel C1. 3, Dark-field illumination of a section of a nontransgenic neonatal heart hybridized to the antisense SV40 polyadenylation sequence probe as a control reveals minimal nonspecific background hybridization. All sections were counterstained with hematoxylin and eosin. All bars indicate 100 μm.

transfected cells in a variety of organ systems. Further, its pan-active properties indicate that it will be useful in the assessment of gene action in transgenic animals in which widespread expression will allow a broad initial survey of biologic activity.

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