Identification of a Transcriptional Enhancer Element Upstream from the Proto-Oncogene *fos*

Abstract. *Sequences upstream from the proto-oncogene* fos *were shown to be essential for its transcription. Transient expression of the chloramphenicol acetyltransferase (CAT) gene linked to upstream sequences of the* fos *gene including its promoter reveals that sequences located 64 to 404 base pairs 5′ to the* fos *cap site contain a typical transcriptional enhancer. Moreover, these enhancer sequences, which are strikingly conserved between mouse and human* fos *genes, coincide with a deoxyribonuclease I–hypersensitive site in the chromatin. The expression of the* fos*-CAT fusion genes was stimulated only two to three times by the* fos *inducer 12-O-tetradecanoyl phorbol-13-acetate. The* fos *enhancer does not appear to be tissue-specific.*

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Efficient transcription of most eukaryotic genes depends on the presence of a transcriptional enhancer (*1*). This element is usually located upstream from the cap site, although it can be found inside the gene. Enhancers can augment the transcription of a gene in a tissue-specific manner (*2*) and are believed to be in certain cases the receptor sites of some cellular regulatory molecules. Because the *fos* proto-oncogene is transiently induced in response to a variety of mitogens and differentiation-stimulating agents (*3*), we were interested in characterizing the sequences essential for its transcription and their involvement in induction.

We linked the 5′ flanking region of the *fos* gene including its promoter to the coding domain of the bacterial chloramphenicol acetyltransferase (CAT) gene (*4*) and assayed for CAT enzymatic activity after transient expression in transfected cells. Figure 1 depicts the structure of the proto-oncogene *fos*, with its promoter and upstream sequences (*5*), the SV40-CAT transcription unit (pSV2CAT) (*4*), and a variety of *fos*-CAT fusion constructs (FC). Transient assays were performed in mouse NIH 3T3 and rat 208F fibroblasts as well as in the human amnion cell line AV-3 and in HeLa cells. The relative CAT activity observed for each construct in the different cell lines is tabulated in Fig. 1.

Construct FC1 contains about 2250 nucleotides of upstream sequences linked to the CAT gene at the Nae I site [map position +41 base pairs (bp), the cap site being defined as +1 bp; Fig. 1]. The CAT activity assayed in extracts from NIH 3T3 cells transfected with FC1 DNA was about 60 percent of that measured after transfection with the control plasmid pSV2CAT, in which the CAT gene is under the control of the SV40

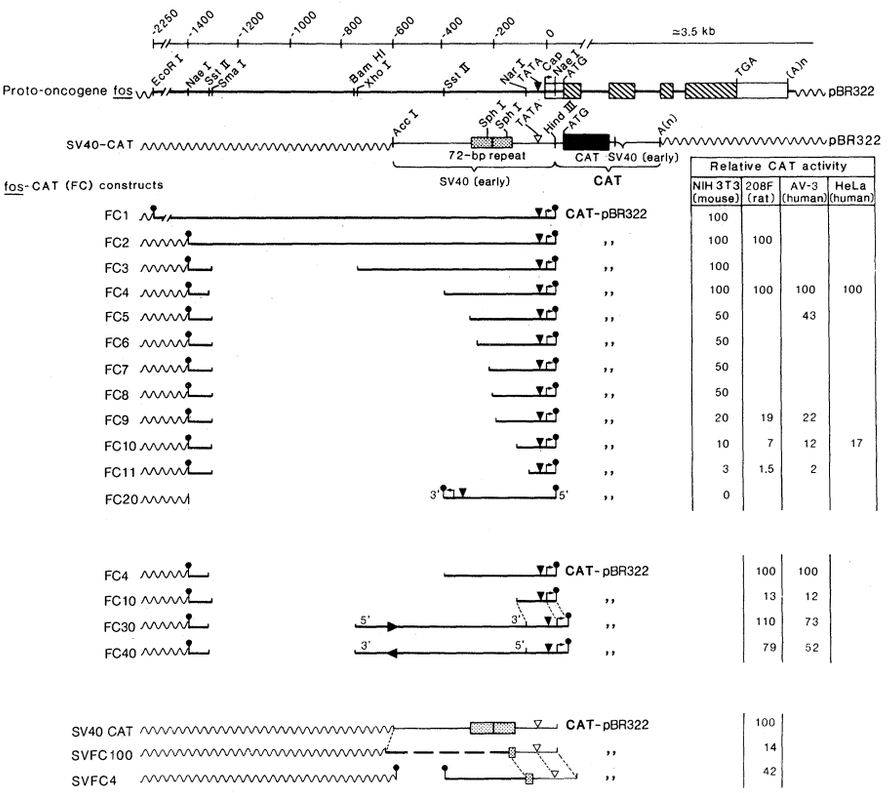

Fig. 1. Structure of the upstream sequences of the human *fos* gene (*5*), SV40-CAT (*4*), and various *fos*-CAT constructs. The enzymatic activity measured in extracts of cells transfected with the *fos*-CAT (FC) and SV40-CAT constructs is tabulated. Standard recombinant DNA procedures were followed (*13*). To generate FC1, we inserted a 2.25-kilobase (kb) Eco RI–Nae I fragment containing upstream sequences and the promoter of the *fos* gene in place of the Acc I–Hind III fragment present in pSV2CAT, by means of Hind III linkers (i). Construct FC2 was generated similarly after Hind III linker ligation to the isolated 1.4-kb Nae I fragment. Deletions from Sma I to Xho I and Sst II to Sst II in construct FC2 yielded constructs FC3 and FC4, respectively. After the deleted fragments corresponding to the residual 5′ flanking sequences and *fos* promoter were digested with Hind III and separated by gel electrophoresis they were recloned in the Sma I–Hind III digested FC2 DNA in place of the original 1.3-kb fragment. The extent of the deletions was determined by agarose gel electrophoresis followed in four cases by direct nucleotide sequence analysis (*14*). The 3′ borders of the deletions map at positions −307 (FC5), −206 (FC9), −124 (FC10), and −64 bp (FC11). We generated construct FC20 by inserting the 404-bp Sst II-Nae I fragment present in construct FC4, in reversed orientation into the Acc I–Hind III–deleted pSV2CAT DNA. We generated constructs FC30 and FC40 by inserting the 671-bp Bam HI–Nar I fragment from construct FC2 in both orientations in the unique Sst II site of construct FC10 after blunt end conversion. We generated construct SVFC4 by replacing the Acc I–Sph I fragment from construct pSV2CAT with the Hind III–Nar I sequences isolated from FC4. We generated construct SVFC100 by inserting a 541-bp Nae I–Sph I fragment originating from the first intron of the *fos* gene in the same Acc I–Sph I-deleted vector as SVFC4. DNA-transfection of NIH 3T3 and 208F cells was carried out by the calcium phosphate precipitation technique (*15*). We transfected AV-3 and HeLa cells by the DEAE-dextran procedure (*16*), using 10 μg of DNA per 10-cm dish. Cell extracts were prepared 48 hours after transfection, and total protein concentration was measured with the BioRad protein assay reagent. CAT assays were performed as described by Gorman *et al.* (*4*). The final concentration of acetyl coenzyme A was 4 m*M*, and 0.5 μCi of [^{14}C]chloramphenicol were used per assay. Under these conditions all reactions were linear for at least 3 hours. As a positive control, pSV2CAT was included in every assay. It showed a variation of not more than 20 percent between experiments. The CAT activity that was observed with constructs SVFC100 and SVFC4 was expressed as a fraction of that corresponding to pSV2CAT.

early promoter and enhancer sequences. The enzymatic activity associated with all other *fos*-CAT hybrids was a fraction of that exhibited by FC1. To identify sequences essential for transcription of the *fos* gene, we made progressive deletions in the upstream region. We generated constructs FC2, FC3, and FC4, in which the 3′ ends of the deletion map at positions −1450, −712, and −404 bp, respectively, by using unique restriction endonuclease sites. The activity of these constructs was similar to that observed with FCl. We obtained a series of plasmids containing more extensive deletions by using Bal 31 exonuclease digestion from the Sst II site of FC4. The 3′ borders of all deletions were determined by restriction mapping, followed by direct nucleotide sequence analysis for FC5, FC9, FC10, and FC11. Constructs FC5, FC6, FC7, and FC8, in which the 3′ ends of the deletions map at positions −307 (FC5) to −220 (FC8) bp, showed about 50 percent of the CAT activity obtained with FC4.

A further 2.5-fold drop in activity was observed with construct FC9, in which the 3′ end of the deletion maps at position −206 bp. The CAT activity declined further in constructs FC10 and FC11, in which the deletion end points reach positions −124 and −64 bp, respectively. Figure 2a shows autoradiographs of the CAT activity of some of the constructs. As a negative control, we generated construct FC20, in which the Sst II–Nae I fragment containing the promoter was reversed (Figs. 1 and 2b). No CAT activity was observed in this construct, an indication that CAT gene transcription depends entirely upon the *fos* gene promoter. The results with the deletion constructs indicated that sequences between −64 and −404 bp were required for efficient transcription of the *fos* gene.

To determine if the upstream sequences needed to augment the transcription of the *fos* gene were analogous to enhancers identified in other eukaryotic genes, we performed two types of experiments. Since an enhancer element is functional in either orientation, we first inserted the 690-bp Bam HI–Nar I fragment in both orientations in the Sst II site of construct FC10, generating constructs FC30 and FC40. We used construct FC10 rather than FC11, because the deletion present in the latter could have impaired the promoter. Both of these constructs gave rise to much higher CAT activity than observed with construct FC10 (Figs. 1 and 2b). CAT gene expression was, however, more efficiently restored if the Bam HI–Nar I fragment was inserted in the original orientation. Sequences upstream from

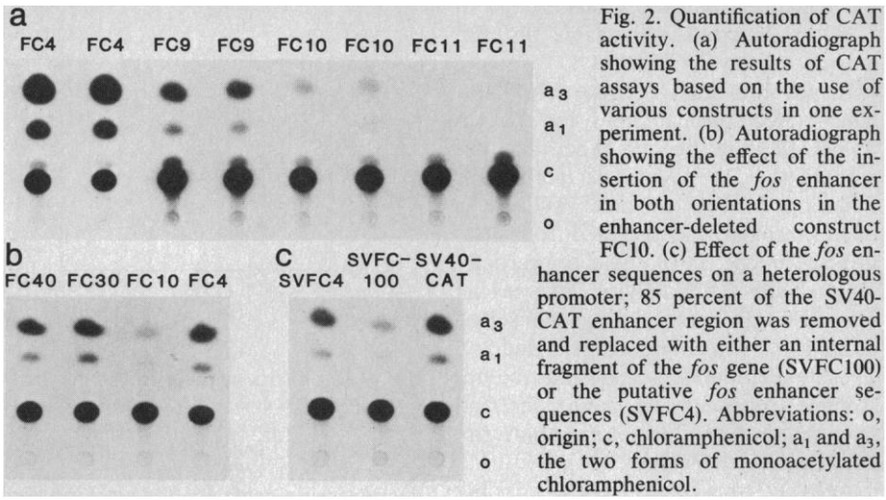

Fig. 2. Quantification of CAT activity. (a) Autoradiograph showing the results of CAT assays based on the use of various constructs in one experiment. (b) Autoradiograph showing the effect of the insertion of the *fos* enhancer in both orientations in the enhancer-deleted construct FC10. (c) Effect of the *fos* enhancer sequences on a heterologous promoter; 85 percent of the SV40-CAT enhancer region was removed and replaced with either an internal fragment of the *fos* gene (SVFC100) or the putative *fos* enhancer sequences (SVFC4). Abbreviations: o, origin; c, chloramphenicol; a_1 and a_3, the two forms of monoacetylated chloramphenicol.

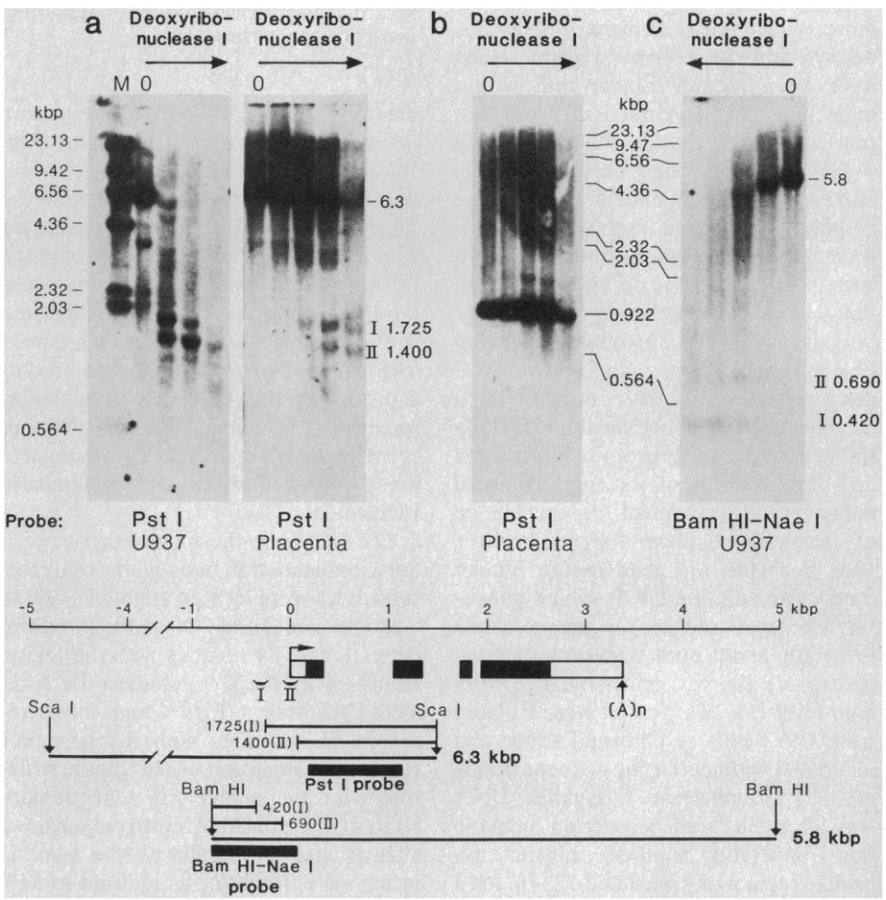

Fig. 3. Mapping of deoxyribonuclease I–hypersensitive sites in the 5′ flanking region of the human *fos* gene. The location of restriction sites and of probes used is indicated in the map at the bottom. The exons of the *fos* gene are indicated by boxes (black, coding region). Arrows marked I and II represent the locations of the hypersensitive sites. (a) Sca I digestions of DNA from U937 or placental nuclei hybridized against a nick-translated probe as indicated; M, Hind III size markers hybridizing with lambda DNA present in probe; O, no deoxyribonuclease I present. (b) The same placental DNA was digested with Pst I and hybridized against the same probe as in (a). (c) DNA from deoxyribonuclease I–treated U937 nuclei was digested with Bam HI and hybridized with the upstream probe. Nuclei were isolated as described (*7*). Cells or minced tissue were homogenized in a buffer containing 0.5*M* sucrose, 0.5 m*M* spermine, 0.15 m*M* spermidine, 60 m*M* KCl, 15 m*M* NaCl, 2 m*M* EDTA, 0.2 m*M* EGTA, 15 m*M* tris, *p*H 7.4. The cells were then centrifuged at 3000*g* and resuspended in the same buffer containing 0.2 to 0.5 percent NP-40. The nuclei were washed by two centrifugation steps in the same buffer with 0.35*M* sucrose and no detergent, and finally suspended in buffer without sucrose. $MgCl_2$ was added to a concentration of 6 m*M*, and digestion was started by the addition of deoxyribonuclease I in increasing amounts (in twofold steps) to the samples and incubation at 14°C for 10 minutes. The digestion was stopped by the addition of sodium dodecyl sulfate, and DNA was then isolated and analyzed.

the enhancer (map positions −2250 to −700 bp) inserted in the same site, did not restore the CAT activity.

Removal of most of the SV40 enhancer (Acc I through Sph I, Fig. 1) from pSV2CAT in the second experiment decreased the CAT synthesis tenfold (*4*). Insertion of *fos* enhancer sequences (Nae I through Nar I isolated from FC4) in place of the SV40 enhancer partially restored the CAT activity, whereas an internal fragment of the *fos* gene inserted at the same site did not (Figs. 1 and 2c). These experiments indicate that the upstream sequences we have identified have features of a prototypic transcriptional enhancer element.

Actively transcribed genes, possibly because of their open configuration, are more sensitive to deoxyribonuclease I digestion than inactive regions. Furthermore, short regions of higher sensitivity, deoxyribonuclease–hypersensitive sites have been identified near and within many genes (*6*). Hypersensitive sites are most frequently found in the 5′ flanking regions of genes, their presence in certain cases being dependent on an active or potentially active state (*7*). Deoxyribonuclease I–hypersensitive sites have been shown to coincide with transcriptional enhancers, including those of globin genes (*7*), immunoglobulin light- and heavy-chain (*8*) genes, and several viral genomes (*9*). We have attempted to establish a relation between the chromatin structure of the proto-oncogene *fos* and the location of its transcriptional enhancer. We examined the occurrence of deoxyribonuclease I–hypersensitive sites near the *fos* gene in the human monocytic cell line U937, which constitutively expressed the *fos* gene at a low level (*10*), and in human placenta, known to express the *fos* gene at a relatively high level (*11, 12*). Nuclei were isolated from U937 cells or placental tissue and incubated with increasing concentrations of deoxyribonuclease I. Isolated DNA was digested with restriction enzyme Sca I and, after Southern transfer, hybridized to a nick-translated 922-bp Pst I fragment (Fig. 3a). Two major deoxyribonuclease I–dependent fragments of approximately 1400 bp (site II) and 1725 bp (site I) can be discerned in both U937 and placental cells. Several minor fragments, which were not consistently detected in different experiments, were also seen.

In this approach, the lengths of the subbands generated should correspond to the distance between the deoxyribonuclease-hypersensitive sites and the Sca I site in the gene. This assumption was valid, since no major hypersensitive

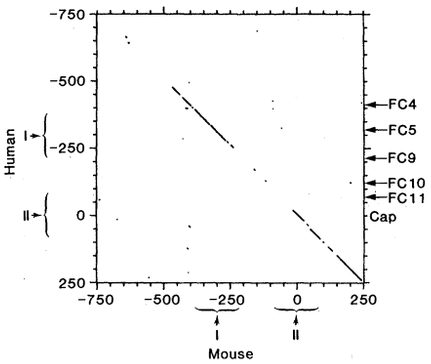

Fig. 4. Dot matrix analysis comparing the 5′ flanking sequences of the mouse (*17*) and human (*5*) *fos* genes. We made the plot, using the "compare" and "dotplot" programs of Devereux *et al.* (*18*), in which strings of 15 nucleotides are compared and a dot is placed whenever at least 12 nucleotides match. The location of the 3′ borders of the deletions of some of the constructs is shown. Arrows marked I and II indicate regions of deoxyribonuclease I hypersensitivity.

sites were detected within the region covered by the probe in placenta (Fig. 3b) or U937 chromatin. Figure 3c shows the result of an experiment in which the same hypersensitive sites were detected with an upstream probe. In this case the DNA isolated from deoxyribonuclease I–treated placental nuclei was digested with Bam HI, and a Bam HI–Nae I fragment (see map of Fig. 3) was used as a probe. In this case also, two major fragments, of about 420 and 690 bp, corresponding to sites I and II, respectively, emerged after deoxyribonuclease I treatment.

The data from these and other experiments indicate that two regions of hypersensitivity were located at about −360 to −240 bp (site I) and −90 to about +80 bp (site II). Site I coincides with the region identified as the *fos* enhancer by transient CAT assays (Figs. 1 and 2), whereas site II coincides with the promoter region. Examination of chromatin structure near the mouse *fos* gene demonstrated the presence of hypersensitive sites at positions similar to sites I and II in human chromatin, in addition to several sites inside the transcription unit.

Sequences essential for efficient functioning of a gene are likely to be conserved through evolution. Figure 4 shows a dot matrix analysis of identities between the promoter and 5′ flanking regions of the human and mouse *fos* genes. The result indicated two regions of striking similarity, one surrounding the cap site and TATA box and another stretching from −250 to −475 bp, the region identified as the transcriptional enhancer. Removal of sequences upstream from position −206 bp (FC9) reduced the activity of the enhancer to 20 percent, whereas deletion upstream from −404 bp did not affect CAT activity. The enhancer sequence identified in the human *fos* gene thus appears to be conserved in the mouse gene.

Our results demonstrate that an element needed for efficient transcription is located upstream from the proto-oncogene *fos*. The precise nature of the essential sequences and the molecular mechanism underlying the enhancer element of the proto-oncogene *fos* remain to be established. The *fos* enhancer does not appear to be tissue-specific according to the CAT assays on different cell types and the results of the deoxyribonuclease I–hypersensitivity experiments.

In response to mitogens or differentiation-specific agents, the *fos* messenger RNA transiently accumulated to levels at least 15 times higher than before induction. The expression of the *fos*-CAT fusion genes was, however, stimulated only two to three times by the *fos* inducer 12-*O*-tetradecanoyl phorbol-13-acetate, values similar in magnitude to those for the induction observed with the Rous sarcoma virus and SV40-CAT constructs. We confirmed these results, using stably transfected cell lines rather than transient assays. These data suggest that either (i) inducer-responsive sequences may be located outside the 2.25-kilobase upstream sequences or possibly within the gene itself or (ii) the mechanism involved might operate either at the transcriptional or at a posttranscriptional level.

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Cloning of a Gene Whose Expression Is Increased in Scrapie and in Senile Plaques in Human Brain

Abstract. *A complementary DNA library was constructed from messenger RNA's extracted from the brains of mice infected with the scrapie agent. The library was differentially screened with the objectives of finding clones that might be used as markers of infection and finding clones of genes whose increased expression might be correlated with the pathological changes common to scrapie and Alzheimer's disease. A gene was identified whose expression is increased in scrapie. The complementary DNA corresponding to this gene hybridized preferentially and focally to cells in the brains of scrapie-infected animals. The cloned DNA also hybridized to the neuritic plaques found with increased frequency in brains of patients with Alzheimer's disease.*

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Scrapie is a naturally occurring slow infection of sheep caused by a class of unconventional infectious agents that have unusual properties. These properties, and the co-purification of a 27- to 30-kilodalton (kD) protein and infectivity, have led to speculation that the enigmatic informational molecule in scrapie is not a nucleic acid (*1–5*). It is now clear, however, that the 27- to 30-kD "prion" protein is the product of a normal cellular gene that is expressed to the same extent in infected and uninfected animals (*6, 7*). The distinctive pathological changes of scrapie are neuronal vacuolation and the formation of abnormal fibrils (*8–12*), but in some animal models of scrapie and cognate diseases (kuru, Creutzfeldt-Jakob disease) there are other pathological alterations that resemble those seen in senile dementia of the Alzheimer type (*13–15*).

We reasoned that whatever the nature of the infectious moiety in scrapie might be, (i) it would use existing cellular biochemical pathways for replication; and (ii) it might induce expression of genes involved in pathological changes common to Alzheimer's disease and scrapie. We therefore looked for messenger RNA's (mRNA's) whose expression was increased in scrapie, and perhaps in Alzheimer's disease, by preparing a complementary DNA (cDNA) library from mRNA's of scrapie-infected mice and then screening the library differentially. Total RNA was isolated by homogenizing (in guanidium isothiocyanate) brains from nine RML Swiss mice infected 5 months earlier with a 10^{-2} dilution of a 10 percent homogenate of brain obtained from mice infected with scrapie (Chandler-adapted strain) (*16*). At the time the animals were killed, they were symptomatic and had titers 10^8 times the median lethal dose per gram of brain. Polyadenylated [poly(A)$^+$] RNA was selected by two cycles of chromatography on oligo(dT)-cellulose (*17*). The cDNA was prepared, as described (*18*), by reverse transcription followed by second strand synthesis with DNA polymerase I. The double-stranded DNA product was digested with S1 nuclease, treated with Eco RI methylase, and ligated to Eco RI dodecamer linkers. After cleavage with Eco RI, DNA larger than 0.6 kilobase was isolated by gel filtration (Sephacryl S1000). The scrapie cDNA (20 ng) was ligated with 1 μg of λgt10 (*19*) cut with Eco RI. The phage was packaged and plated on a lawn of BNN104 (C600r−M$^+$hflA150). Phage λgt10 is lysogenic in hfl (high-frequency lysogeny) strains, and insertion of DNA into the single Eco RI site of the cI λ repressor gene yields recombinants that form clear plaques on BNN104 (*19*).

About 7 percent of the packaged phage were recombinants, and more than 99 percent of 365,000 independent recombinants contained inserts. The library was amplified in BNN104, and 5000 recombinant phage were picked and transferred to fifteen 15-cm plates for screening. Replicas were made from each plate on nylon filters (Pall Biodyne) in accordance with the manufacturer's protocols. The replicate filters were acetylated (*20*) and prehybridized. For differential screening, one set of filters was hybridized to ^{32}P-labeled cDNA that was reverse-transcribed from poly(A)$^+$ RNA of scrapie-infected brain, with oligo(dT) used as primer. The other set of filters

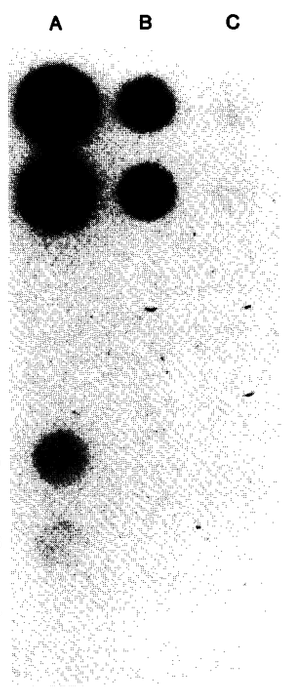

Fig. 1. Dot-blot hybridizations of RNA with Scr-1 DNA probe. Lanes A, B, and C contain, respectively, 1, 0.2, and 0.05 μg of poly(A)$^+$ RNA, or five times as much total RNA. RNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled Scr-1 DNA as described (*23*). (1) Poly(A)$^+$ RNA from brains of scrapie-infected mice; (2) total RNA from infected mice; (3) wheat germ RNA; (4) calf thymus RNA; (5) poly(A)$^+$ RNA from brains of uninfected mice; and (6 and 7) total RNA from brains of uninfected animals from two sources.