16. Hamster hind-leg skeletal muscle and the smooth-muscle portion of esophagus were removed and rinsed in ice-cold 0.9% NaCl. Muscles were homogenized in 260 m*M* sucrose, 50 m*M* Hepes, pH 7.4, through the use of a Brinkmann Polytron at half-maximal setting for 30 seconds. Homogenates were centrifuged at 1000*g* for 10 minutes. The supernatant was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 20 minutes. The supernatant was centrifuged at 100,000*g* for 60 minutes. The resulting pellet was used for [^{3}H]nitrendipine binding assays as above. Other smooth muscles including bladder were prepared as above and assayed for [^{3}H]nitrendipine binding. These tissues also displayed the same trend of increased binding in the CM hamsters compared with controls.
17. Tissue for (−)desmethoxyverapamil binding was prepared as described (*14*). Protein (0.5 to 1.0 mg) was added to tubes containing 0.1 to 200 n*M* (−)[^{3}H]desmethoxyverapamil (a gift from Knoll AB, Ludwigshafen, FRG, original specific activity 83 Ci/mmol) in the presence or absence of 1 μ*M* methoxyverapamil. After 1 hour at room temperature (22° to 24°C) reactions were terminated by filtration over glass fiber filters pretreated with 0.5% polyethyleneimine to reduce nonspecific filter binding. Membranes were washed and trapped radioactivity determined as above.
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19. Synaptosomes were prepared by the method of Hajos [*Brain Res.* **93**, 485 (1975)]. Membranes, removed from the 0.32 to 0.8*M* sucrose interface, were diluted by 2.5- to 3-fold with nondepolarizing (basal) buffer (composition, m*M*: NaCl, 145; KCl, 5; $CaCl_2$, 1.2; $MgCl_2$, 1.2; Hepes, 10: *p*H adjusted to 7.4 with Trizma) and centrifuged at 9,000g for 20 minutes. The pellet was resuspended in basal buffer and preincubated at 30°C for 20 minutes. Uptake of $^{45}Ca^{2+}$ was initiated by addition of 100-μl aliquots of tissue (1 to 2 mg of protein) to 0.9 ml basal or stimulating (in which 57.5 m*M* KCl was iso-osmotically substituted for NaCl) buffer containing $^{45}Ca^{2+}$. Reactions were terminated after 10 seconds by the addition of 4 ml of ice-cold basal buffer followed by filtration over glass fiber filters. Trapped radioactivity was assessed as above. Voltage-stimulated uptake was defined as the difference between accumulated $^{45}Ca^{2+}$ in the presence of stimulating and basal buffer.
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Gene Transfer and Molecular Cloning of the Human NGF Receptor

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Nerve growth factor (NGF) and its receptor are important in the development of cells derived from the neural crest. Mouse L cell transformants have been generated that stably express the human NGF receptor gene transfer with total human DNA. Affinity cross-linking, metabolic labeling and immunoprecipitation, and equilibrium binding with ^{125}I-labeled NGF revealed that this NGF receptor had the same size and binding characteristics as the receptor from human melanoma cells and rat PC12 cells. The sequences encoding the NGF receptor were molecularly cloned using the human Alu repetitive sequence as a probe. A cosmid clone that contained the human NGF receptor gene allowed efficient transfection and expression of the receptor.

NERVE GROWTH FACTOR (NGF) and its receptor play an essential role in the survival and maintenance of sympathetic and sensory neurons. NGF action is initiated by the interaction of NGF with a specific receptor on the neuronal cell surface. Receptors for NGF have also been found on nonneuronal derivatives of the neural crest, including melanoma cells (*1, 2*), pheochromocytoma cells (*3*), Schwann cells, and neurofibroma cells (*2*). The NGF receptor has been characterized in sympathetic nerve cells (*4*), pheochromocytoma (PC12) cells (*3, 5, 6*), and melanoma cells (*2, 7*). Immunoprecipitation and photoaffinity cross-linking studies indicate that it has a molecular size of 70,000 to 80,000 daltons (*2, 7*) and can also exist in a dimeric form of about 200,000 daltons (*2, 5, 7*). The receptor can occur in both high and low affinity forms (*3, 8*). Further characterization of the human receptor indicates that it is a glycoprotein and is phosphorylated on serine resides (*9*).

DNA-mediated gene transfer has been used to express and clone the human NGF receptor. Unlike other techniques, this method does not depend on messenger RNA (mRNA) enrichment, but requires the availability of an appropriate recipient cell line and a means of identifying cells which have taken up and expressed the gene. To screen the recipient cells, we used an immunological rosette assay in which cells are incubated with monoclonal antibody to human NGF receptor (*2*) and then with second antibody [rabbit antibody to mouse immunoglobulin G (IgG)] coupled to erythrocytes. This assay is quite sensitive since rosettes can be observed with the human neuroblastoma cell line SY5Y, which expresses fewer than 1000 receptors per cell (*10*). When mouse fibroblast L cells are assayed in the same manner no rosettes are observed.

High molecular weight DNA was isolated from human neuroblastoma SY5Y, human melanoma A875 cells, or human T cells and mixed with the purified herpesvirus thymidine kinase (pTK) gene to produce a calcium phosphate precipitate for transformation of mouse fibroblast L cells (Ltk$^-$) (*11*). After 2 weeks in HAT [hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), and thymidine (5 μg/ml) selection medium, the tk$^+$ colonies were screened with the rosette assay (*12*). Positive colonies were observed at a frequency of 1 per 12,000 tk$^+$ colonies with DNA from all three cell types. The positive L cell colonies were isolated with cloning cylinders and purified by two rounds of rescreening with the rosette assay. The primary transformants were then grown in bulk, and four clones were analyzed further for the presence of NGF receptor (see below). HAT selection was maintained throughout the purification and growth of the cells. The transformant lines were stable over many generations and did not lose the foreign NGF receptor gene or display decreased receptor expression.

To reduce the amount of extraneous human DNA, one of the primary transformants was transferred through a second round of transformation. The positive cell line N21, which was generated from human neuroblastoma DNA, was used as the source of DNA for a second round of transformation. High molecular weight DNA was isolated from N21 and used with pTK for transformation into mouse Ltk$^-$ cells. A secondary transformant, N21-11, was isolated with HAT selection and the rosette assay.

We used affinity cross-linking with ^{125}I-labeled NGF (*5–7*) to test for NGF receptor in the L cell transformants that had been identified by the rosette assay. Cells in suspension were incubated with ^{125}I-labeled NGF at 0°C and then treated with ethyldimethylisopropylcarbodiimide to cross-link

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the bound NGF to the NGF receptor. The cells were then solubilized, the receptor was immunoprecipitated with a monoclonal antibody (ME20.4) to the NGF receptor (*2*), and the immunoprecipitate was subjected to electrophoresis. In agreement with earlier cross-linking studies, the ^{125}I-labeled NGF–NGF receptor complex from the melanoma cell line Hs294 had an apparent molecular size of approximately 100,000 daltons and was immunoprecipitated by ME20.4 (Fig. 1A, lanes g and h). No labeled material was seen with the mouse fibroblast Ltk$^-$ cell line (Fig. 1A, a and b) because L cells do not express the murine NGF receptor. The primary transformant E9b and the secondary transformant N21-11 contained a cross-linked ^{125}I-labeled NGF–NGF receptor complex of 100,000 daltons, which was immunoprecipitated by the specific antibody ME20.4. An estimated molecular weight for the receptor of 75,000 was calculated by subtracting the molecular weight of the β dimer of NGF from that of the complex. This is in agreement with the molecular weight reported for the human NGF receptor (*5, 7, 9*). Each cell line identified by the rosette assay with the anti–NGF receptor monoclonal antibody produced a cross-linked ^{125}I-labeled NGF–NGF receptor complex of the same molecular size. The expression of the NGF receptor in the transformants was not due to an activation of the endogenous mouse NGF receptor because the monoclonal antibody recognizes only the human NGF receptor molecule.

Expression of the NGF receptor in the transformant mouse L cells was also detected by labeling the cells with [^{35}S]cysteine in the presence and absence of tunicamycin. After extraction with detergent the NGF receptor was immunoprecipitated and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and fluorography. The three primary transformant lines contained different amounts of NGF receptor (Fig. 1B). No NGF receptor was detected in mouse Ltk$^-$ cells labeled in a similar manner. The melanoma cell line A875 contained a 70,000-dalton species, identical to that seen in the three primary transformants, E9b, E4b, and E3a.

Treatment of human melanoma cells with tunicamycin blocks *N*-linked glycosylation of the NGF receptor precursor, yielding a receptor peptide with a reduced apparent molecular size of 62,000 daltons (*9*). The receptor from the primary transformant cell lines showed similar reductions in apparent size (Fig. 1B). Thus the NGF receptor in primary transformants is *N*-glycosylated to the same extent as the receptor in human melanoma cells.

Each transformant specifically bound

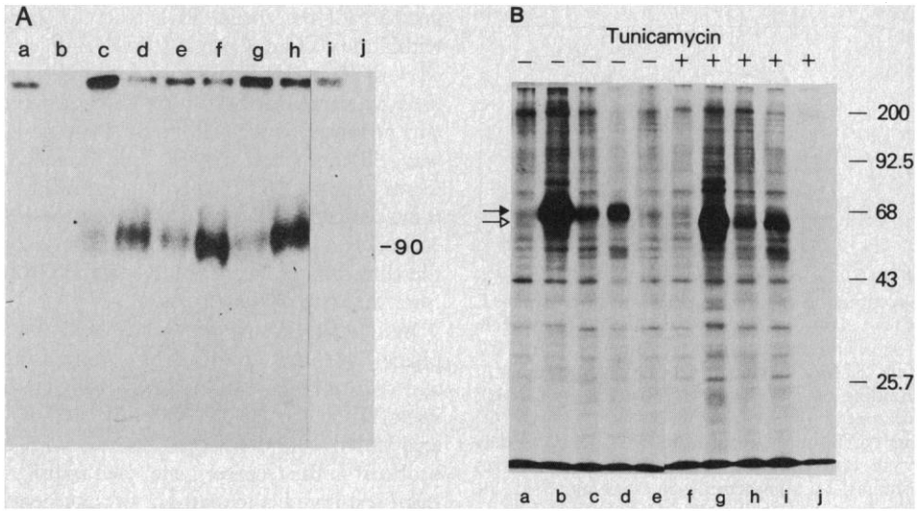

Fig. 1. (A) NGF receptors on transformants demonstrated by affinity labeling and immunoprecipitation. After affinity labeling, cell extracts were immunoprecipitated and then prepared for electrophoresis by boiling with an equal volume of SDS sample buffer containing 10% 2-mercaptoethanol. (Lanes a, c, e, and g) Cell homogenates prior to immunoprecipitation; (lanes b, d, f, and h) immunoprecipitates obtained with the IgG fraction of ME20.4; (lanes i and j) control immunoprecipitates with an IgG from an irrelevant monoclonal antibody C10-7; (lanes a and b) mouse L cells; (lanes c, d, and i) secondary transformant N21-11; (lanes e, f, and j) primary transformant E9b; (lanes g and h) melanoma Hs294. (B) Cells were labeled with [^{35}S]cysteine for 16 hours in fresh medium lacking cysteine and some cultures were incubated with tunicamycin (10 μg/ml) (Calbiochem) both for the 2 hours before and during labeling with [^{35}S]cysteine. The NGF receptor was extracted from the labeled cells, immunoprecipitated with the monoclonal antibody ME20.4, and analyzed by SDS gel electrophoresis (*2*). (Lanes a and f) Ltk$^-$ cells; (lanes b and g) human melanoma A875; (lanes c and h) transformant lines E3a; (lanes d and i) transformant line E9b; (lanes e and j) transformant line E4b. (Closed arrow) NGF receptor; (open arrow) NGF receptor synthesized in the presence of tunicamycin. A longer exposure of this gel indicated the presence of the receptor in lanes e and j.

NGF (Fig. 2). As expected from the cross-linking and immunoprecipitation data, mouse L cells did not bind any labeled NGF specifically. For both E9b and N21-11 cell lines, the Scatchard plots for the transformant cell lines were linear, indicating one class of receptors. Although E9b cells had five times as many receptors per cell as N21-11 cells, the equilibrium dissociation constant was the same, approximately 1 n*M*.

Dissociation of ^{125}I-labeled NGF from receptors on sympathetic and sensory neurons, and the PC12 cell line revealed a high and low affinity class of binding which differed 100-fold in dissociation rate. Human melanoma A875 cells have mainly the rapidly dissociating receptor class (*5, 13*) while human neuroblastoma SY5Y cells have only the slower dissociating (high affinity) class (*10*). Dissociation of ^{125}I-labeled NGF from mouse transformant cell lines indicated a single kinetic class of receptor molecules with a rate constant comparable to the rapidly dissociating NGF receptor in PC12 cells and A875 melanoma cells.

To identify human DNA present in the transformed murine cells, we used hybridization to highly repetitive Alu sequences (*14*). Because the human genome contains at least 300,000 copies of widely interspersed repetitive sequences (*15*), nearly every human gene is in close proximity to one or more Alu repetitive sequences. The 300-bp Alu sequence was used as a molecular probe in Southern blot analysis (*16*) of DNA isolated from the transfectants.

The human Alu probe did not cross-hybridize to any mouse cellular sequences in Ltk$^-$ DNA (Fig. 3). Two primary transformants that express the NGF receptor had many sequences that hybridized to the nick-translated Alu probe, whereas the secondary transformant, N21-11, had a much simpler pattern (Fig. 3, lane c). The total size of Alu-positive bands in the DNA from the secondary transfectant (digested with Bam HI and other restriction enzymes) ranged from 10 to 15 kilobases (kb). For comparison, DNA isolated from a secondary NIH/3T3 transformant containing the *ras* oncogene transferred from human melanoma DNA (*17*) had far more human DNA sequence, as assayed by the Alu probe (Fig. 3, lane e).

If the human NGF receptor gene was closely linked to one of the Alu repetitive sequences, the gene could be isolated by molecularly cloning the Alu positive clones from a library of transformant DNA. Therefore, a lambda genomic library (*18*) was prepared with N21-11 DNA partially cleaved with Eco RI. One Alu-positive phage (λR1) was identified in 100,000

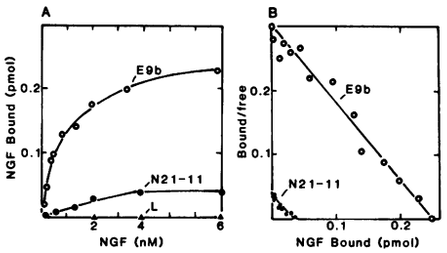

Fig. 2. Equilibrium binding analysis of NGF receptors on transformed cell lines. (A) Mouse L cells and transformed derivatives N21-11 and E9b were harvested in phosphate-buffered saline, 0.2 m*M* EDTA, bovine serum albumin (1 mg/ml). Binding of ^{125}I-labeled NGF to cells (1.5×10^6 cells per milliliter) was assayed in triplicate (*2*) after a 90-minute incubation at 0°C. Binding data were corrected for nonspecific (nonsaturable) binding by subtracting values obtained in the presence of unlabeled NGF (5 μg/ml). Values are plotted in terms of moles of NGF bound per 2.25×10^5 cells. (B) The data in (A) plotted according to Scatchard.

phage recombinants screened with nick-translated Alu fragment. This phage contained a 14-kb Eco RI insert and the Alu sequence was mapped within a 3.8-kb Bam HI restriction fragment (Fig. 4).

To obtain overlapping sequences complementary to λR1, the 5-kb Bam HI restriction fragment adjacent to the 3.8-kb fragment from λR1 (Fig. 4) was nick-translated and used to probe the partial Hae III–Alu human genomic Charon 4A library (*19*). Three phages were isolated that hybridized with the 5-kb Bam HI fragment (Fig. 4). Each of these phages was tested by cotransformation in mouse L cells and was found negative by rosette assay. This result indicated that no single phage contained the intact receptor gene (Table 1). Combinations of these phages were used for transformation to test whether the intact gene might be reconstituted by homologous recombination between phages with overlapping regions (*20*). Positive rosettes were seen when λR9 was mixed with any one of the three other phages. No combination of phages lacking λR9 gave any positive rosettes in the transformation assay. The number of positives was approximately 1 per 1000 Ltk$^+$ transformants.

Cosmid clones that overlapped the four recombinant phage inserts were isolated by screening a human cosmid library (*21*) with the 5-kb Bam HI restriction fragment. This library contains 30- to 45-kb fragments of human leukocyte DNA in the cosmid vector pJB8, which contains the herpesvirus thymidine kinase gene. One of the two cosmids isolated, C3, was characterized in detail (Fig. 4) and used to transform mouse Ltk$^-$ cells. Positive rosettes were formed at a rate of 30 percent (Table 1), consistent with the presence of the entire NGF receptor gene within the cosmid. Cosmid C3 DNA digested with Bam HI, Eco RI, or Hind III and used to transform Ltk$^-$ cells did not yield any positive rosettes although the cosmid was still competent to yield Ltk$^+$ transformants (Table 1). The lower number of Ltk$^+$ transformants with the Eco RI–digested C3 cosmid is attributed to the interruption of the tk promoter at an Eco RI site −87 bp from the start of transcription.

Individual transformants obtained with phage DNA were purified by rosette assay and cellular DNA was isolated from these lines. When the DNA's were digested and analyzed by Southern blot analysis with a probe from the receptor gene, each transformant exhibited the pattern of fragments expected from the phage maps in Fig. 4. Evidence that the intact receptor gene was reconstituted during the transformation also came from affinity cross-linking experiments. Receptor-positive L cell lines, originating from transfections with the recombinant phages, yielded a cross-linked species the same size as that found in PC12 cells and human melanoma cells. Immunoblotting of the NGF receptor from the phage transformant lines also indicated that the receptor is of the expected size. Thus partially overlapping phage DNA's containing receptor sequences can undergo recombination during DNA-mediated gene transfer to generate an apparently intact receptor gene.

Southern blot analysis of the NGF receptor gene in human DNA indicated that there was a single copy of the gene. The same 5-kb Bam HI restriction fragment could be visualized at roughly the same intensity in A875 cells, Josh cells (a pre-B cell line), the three independent primary transformants, and the N21-11 secondary transformant (Fig. 5A). Hence the high level of expression of the NGF receptor by A875 cells and the E9b transformant was not due to an amplification of the NGF receptor gene.

Table 1. Frequency of positive colonies after transformation. Transformation of mouse L cells with recombinant phages and cosmid clones was carried out as described (*11*) except that 1 μg of phage or cosmid DNA was included with 100 ng of pTK and 15 μg of Ltk$^-$ DNA in the calcium phosphate precipitate. The pTK was omitted from the cosmid transfections. After 2 weeks in HAT selection medium the plates were screened by the rosette assay (*12*) and then stained with Giemsa to determine the total number of Ltk$^+$ colonies.

DNA source	Colonies screened	Positives
λR1	5800	0
λR5	2700	0
λR9	2600	0
λR11	4800	0
λR1 + λR9	1600	2
λR5 + λR9	2100	3
λR11 + λR9	2700	1
λR1 + λR5	3000	0
λR1 + λR11	3100	0
λR5 + λR11	4500	0
C3	290	98
C3 (Bam HI)	240	0
C3 (Eco RI)	35	0
C3 (Hind III)	210	0

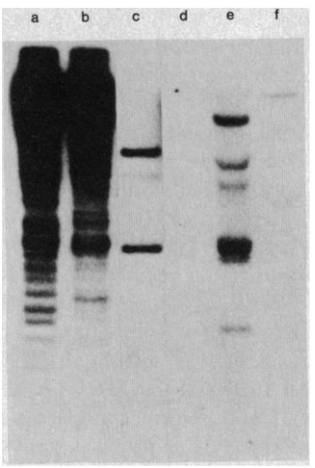

Fig. 3. Human repetitive Alu sequences in transformed cell lines. Total cellular DNA was isolated, digested with Bam HI, and separated on a 1% agarose gel. After transfer to nitrocellulose, the DNA (20 μg/lane) was probed with the 300-bp Alu repetitive sequence. (Lane a) Primary transformant E9b; (lane b) primary transformant N21; (lane c) secondary transformant N21-11; (lane d) mouse Ltk$^-$; (lane e) secondary NIH/3T3 transformant containing human *ras* sequences (*17*); (lane f) ^{32}P-labeled λ DNA digested with Hind III.

The size and the abundance of mRNA encoding the human NGF receptor was examined in polyadenylated RNA populations from the L cell transformants and A875 cell line. Isolated RNA's were separated electrophoretically, transferred to nitrocellulose, and probed with the 5-kb Bam HI genomic fragment from λR1. A single 4-kb mRNA was detected in E9b, N21, N21-11, and A875 RNA but not in mouse L cell RNA (Fig. 5B). The amount of NGF receptor mRNA paralleled the amount of cell-surface NGF receptor measured by equilibrium binding. In particular, the E9b cell line, which expresses more than 500,000 NGF receptors per cell, contained more receptor mRNA than the N21-11 line and its parent line, N21 (Fig. 5B, lanes d and e). The same 4-kb receptor mRNA was detected in the SY5Y neuroblastoma cell line, although much more polyadenylated RNA was required for detection (Fig. 5B, lane g). The presence of the same mRNA in SY5Y cells as in A875 cells suggests that both the high and the low affinity receptors are encoded by the same gene. Thus there is a single 4-kb NGF receptor mRNA in human

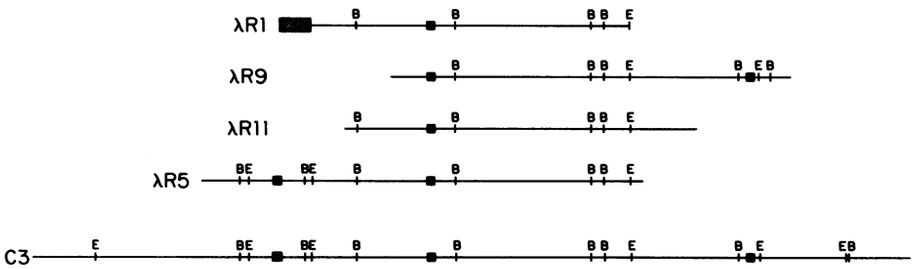

Fig. 4. Restriction maps of NGF receptor genomic clones used in transformation. λR1 was cloned from a library made from N21-11 DNA. λR5, λR9, and λR11 were isolated from the Hae III–Alu human genomic library (*19*) and C3 was isolated from a human cosmid library (*21*). The striped box represents mouse cellular sequences and the black boxes represent human Alu sequences. Oligonucleotide probes complementary to the cohesive ends of lambda DNA (New England Biolabs) were end-labeled with [^{32}P]ATP and T4 polynucleotide kinase, and used to map restriction sites.

melanoma and neuroblastoma cells and in the mouse L cell transformants.

This contrasts with the heterogeneity of functional and biochemical forms of the NGF receptor. However, the low and high affinity species of the receptor must be structurally related because a monoclonal antibody to the NGF receptor recognizes both the A875 receptor, which is exclusively the low affinity form (*2*), and the SY5Y receptor, which is exclusively high affinity (*10*). In addition, the NGF receptor gene hybridizes to the same 4-kb mRNA in both cell lines.

Receptors for NGF in the PC12 and A875 cell lines are 70,000- to 80,000-dalton monomers which can dimerize to form a 200,000-dalton receptor (*7*, *9*, *13*). In addition, Hosang and Shooter (*6*) have observed a 158,000-dalton NGF–NGF receptor complex resulting from affinity cross-linking experiments in PC12 cells. The relation of this intermediate species to the other receptor species is unclear.

We examined the binding of NGF in the transformed mouse fibroblasts in both kinetic and steady state experiments, and found that the receptor expressed was almost exclusively of the low affinity type. This form of the receptor on the mouse fibroblasts resulted from transformations with DNA from cells with high affinity NGF receptors, as well as from cells with mainly low affinity receptors. The receptor in transformants was 70,000 to 80,000 daltons and indistinguishable from receptors on PC12 cells and human melanoma cells. It has been suggested that only the high affinity form of NGF receptor can mediate functional responses to NGF (*22*). If this is the case, the L cell transformants, which have only low affinity receptors, may not be responsive to NGF.

Thus, we have isolated a cosmid DNA clone that spans the NGF receptor gene and that produces NGF receptor expression in mouse L cells with high efficiency. This cosmid can be used to introduce the NGF receptor into various cell types and to determine the necessary cellular environments for expression of the high affinity receptor. These studies may also indicate the characteristics required to render a cell competent to respond to NGF.

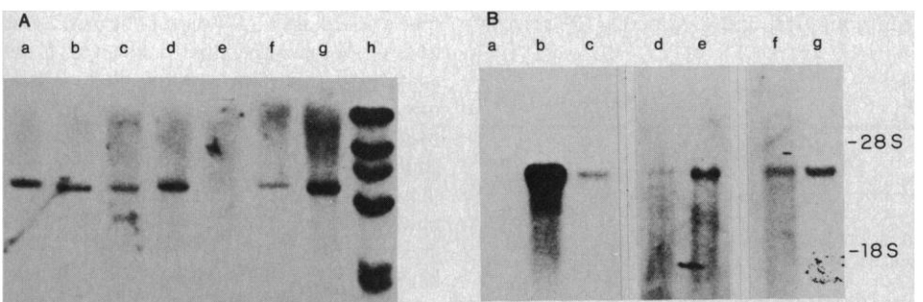

Fig. 5. (A) Southern blot analysis of high molecular weight DNA from transformed cell lines and human DNA. A sample (20 μg) of DNA (except for lane f where 10 μg was used) was digested with Bam HI, fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a 5-kb Bam HI genomic fragment from λR1. (Lane a) Transformant E4b; (lane b) transformant E9b; (lane c) N21; (lane d) N21-11; (lane e) Ltk$^-$; (lane f) A875; (lane g) Josh; (lane h) labeled lambda DNA digested with Hind III. (B) Northern blot analysis of polyadenylated RNA from L-cell transformants and human A875 and SY5Y cell lines. Each panel represents a different experiment. (Lane a) Five micrograms of Ltk$^-$ RNA; (lane b) 5 μg of E9b RNA; (lane c) 2 μg of A875 RNA; (lane d) 5 μg of N21 RNA; (lane e) 5 μg of N21-11 RNA; (lane f) 1 μg of E9b RNA; (lane g) 30 μg of SY5Y RNA.

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18. Total cellular DNA from the secondary transformant, N21-11, was isolated and partially digested with Eco RI. After fractionation on a 0.6% agarose gel, fragments ranging from 12 to 20 kb were excised and eluted from the gel. The fragments were mixed with Eco RI–digested arms of λgtWES [P. Leder, D. Tiemeir, L. Enquist, *Science* **196**, 175 (1977)], incubated with T4 DNA ligase, and packaged in vitro. The library was screened using the 300-bp Alu restriction fragment, which had been excised and purified by gel electrophoresis from Blur8 plasmid digested with Bam HI (*15*).
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