A Protein Induced During Nerve Growth (GAP-43) Is a Major Component of Growth-Cone Membranes

J. H. PATE SKENE, RICHARD D. JACOBSON, G. JACKSON SNIPES, C. BRIAN MCGUIRE, JEANETTE J. NORDEN, JOHN A. FREEMAN

Growth cones are specialized structures that form the distal tips of growing axons. During both normal development of the nervous system and regeneration of injured nerves, growth cones are essential for elongation and guidance of growing axons. Developmental and regenerative axon growth is frequently accompanied by elevated synthesis of a protein designated GAP-43. GAP-43 has now been found to be a major component of growth-cone membranes in developing rat brains. Relative to total protein, GAP-43 is approximately 12 times as abundant in growth-cone membranes as in synaptic membranes from adult brains. Immunohistochemical localization of GAP-43 in frozen sections of developing brain indicates that the protein is specifically associated with neuropil areas containing growth cones and immature synaptic terminals. The results support the proposal that GAP-43 plays a role in axon growth.

GAP-43 IS ONE OF A SMALL GROUP of axonally transported "growth-associated" proteins whose synthesis is increased 20- to 100-fold during successful regeneration of axons in the central nervous system (CNS) of nonmammalian vertebrates (*1–4*) and in peripheral nerves of mammals (*5*). In adult mammalian CNS pathways that do not regenerate, GAP-43 synthesis and transport fails to increase beyond low background levels in response to injury (*5, 6*). During mammalian CNS development, however, GAP-43 is initially synthesized at a high level throughout the brain; this synthesis declines with increasing age (*6–9*). The relation between high levels of GAP-43 synthesis and successful axon growth suggests that the protein might directly participate in one or more steps in axon elongation or synapse formation.

Many steps in axonal growth, including membrane addition and recognition of substrata and target cells, occur at specialized motile structures called "growth cones" (*10–12*). A possible association of GAP-43 with growth cones was suggested by the accumulation of newly synthesized GAP-43 in the distal segments of regenerating toad optic nerves (*13*). To further examine the subcellular distribution of GAP-43 in the developing CNS, we isolated growth cones from homogenates of fetal (embryonic day 18) or neonatal (day 3) rat brains by the discontinuous sucrose gradient method (*14*). Brains were homogenized in a solution containing 0.32*M* sucrose, 1 m*M* *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES) (*p*H 7.3), and 1 m*M* $MgCl_2$ and centrifuged at 1500*g* for 15 minutes. The low-speed supernatant was layered onto a discontinuous sucrose gradient (steps of 0.75, 1.0, and 2.66*M* sucrose in 1 m*M* of TES at *p*H 7.3 and 1 m*M* of $MgCl_2$) and centrifuged at 242,000*g* for 40 minutes (Beckman VTi50 rotor). The fraction at the interface of the load and the 0.75*M* sucrose step (growth cones) was recovered. Electron microscopic examination of this fraction (Fig. 1) showed that the isolated growth-cone preparation was essentially homogeneous, consisting of membrane-bound elements resembling those analyzed by Pfenninger *et al.* (*14*).

Proteins in a crude membrane fraction derived from the growth-cone preparation were compared by two-dimensional (2-D) gel electrophoresis with membrane proteins from regenerating toad optic nerves containing GAP-43. Portions of the growth-cone fraction were diluted to a ratio of 1:1 with a lysis buffer consisting of 1 m*M* TES at *p*H 7.3, 1 m*M* $MgCl_2$, and 0.5 m*M* $CaCl_2$, stirred 15 minutes on ice, and centrifuged for 30 minutes at 100,000*g* to recover membranes. The pellets were resuspended in 1 m*M* TES at *p*H 7.3 with 1 m*M* $MgCl_2$; portions for electrophoresis were brought to 0.5% SDS and 5 m*M* dithiothreitol (DTT). To prepare the samples of regenerating nerves, toads (*Bufo marinus*) were anesthetized on ice, and their left optic nerves were

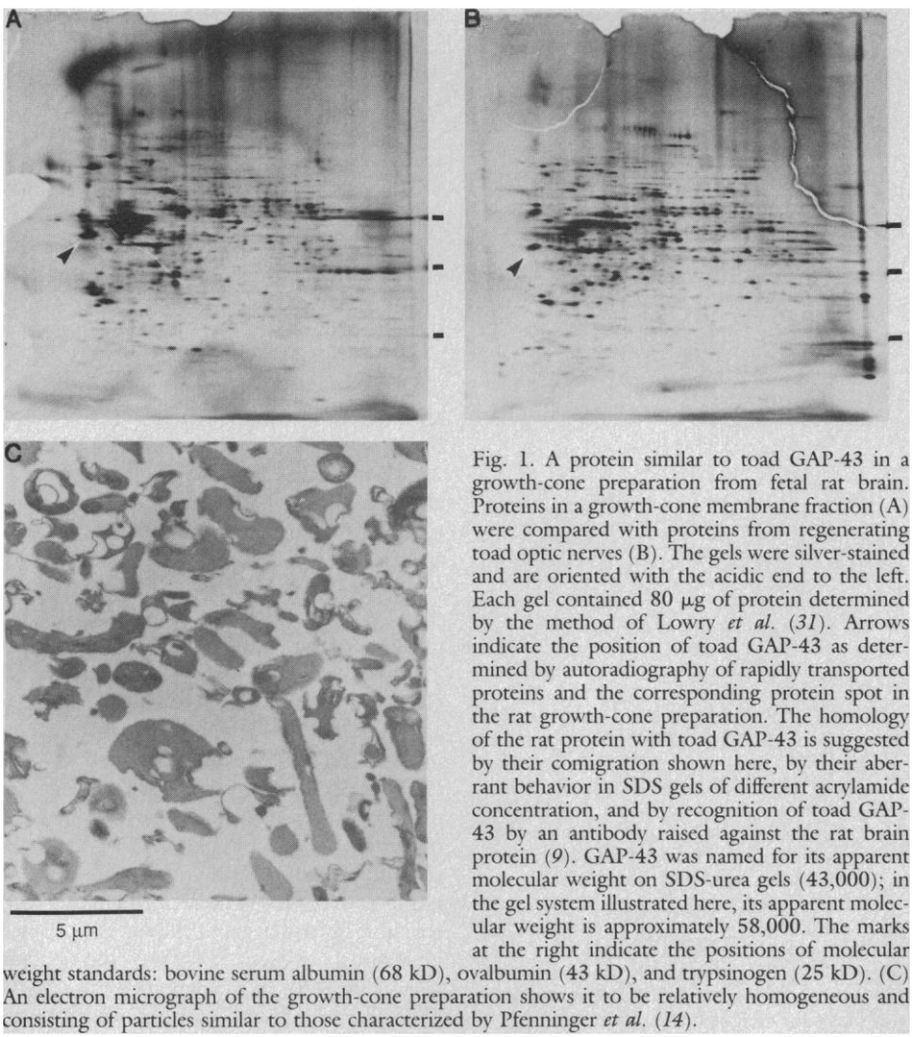

Fig. 1. A protein similar to toad GAP-43 in a growth-cone preparation from fetal rat brain. Proteins in a growth-cone membrane fraction (A) were compared with proteins from regenerating toad optic nerves (B). The gels were silver-stained and are oriented with the acidic end to the left. Each gel contained 80 μg of protein determined by the method of Lowry *et al.* (*31*). Arrows indicate the position of toad GAP-43 as determined by autoradiography of rapidly transported proteins and the corresponding protein spot in the rat growth-cone preparation. The homology of the rat protein with toad GAP-43 is suggested by their comigration shown here, by their aberrant behavior in SDS gels of different acrylamide concentration, and by recognition of toad GAP-43 by an antibody raised against the rat brain protein (*9*). GAP-43 was named for its apparent molecular weight on SDS-urea gels (43,000); in the gel system illustrated here, its apparent molecular weight is approximately 58,000. The marks at the right indicate the positions of molecular weight standards: bovine serum albumin (68 kD), ovalbumin (43 kD), and trypsinogen (25 kD). (C) An electron micrograph of the growth-cone preparation shows it to be relatively homogeneous and consisting of particles similar to those characterized by Pfenninger *et al.* (*14*).

J. H. P. Skene and R. D. Jacobson, Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305.
G. J. Snipes, C. B. McGuire, J. J. Norden, J. A. Freeman, Department of Cell Biology, Vanderbilt University Medical School, Nashville, TN 37232.

exposed intracranially and crushed with jewelers' forceps near the point at which the nerve enters the cranium. Two weeks later the toads were again anesthetized, and their left eyes were injected with 400 μCi of [^{35}S]methionine (Amersham). After 4 to 6 hours, the toads were killed, and their left optic nerves and right optic tracts were removed and homogenized in 10 m*M* tris at *p*H 7.5, 5 m*M* DTT, and 5 m*M* EDTA. The membranes were pelleted as above and redissolved in 0.5% SDS and 5 m*M* DTT. The samples for electrophoresis were heated to 95°C for 5 minutes, cooled, and mixed with an equal volume of 8*M* urea, 10% nonidet-P40, and 5 m*M* DTT. Two-dimensional electrophoresis was carried out essentially according to the method of O'Farrell (*15*). Samples were applied to isoelectric focusing gels (1.2-mm diameter tubes) containing 4% acrylamide, 0.21% bisacrylamide, 9*M* urea, 1.6% ampholytes (LKB Instruments) at *p*H 3.5 to 10, and 0.8% ampholytes at *p*H 5 to 7 and focused at 350 V for 18 hours. SDS gel electrophoresis in the second dimension was on gels containing a linear gradient of 5 to 15% acrylamide in the buffer system of Laemmli (*16*). Gels were first fixed and stained with Coomassie blue (*17*) and then silver stained (*18*). The position of authentic GAP-43 (*1*) was determined by exposing the gel containing radioactive axonally transported toad proteins to x-ray film. A protein electrophoretically and antigenically similar to GAP-43 from the regenerating optic nerve of the toad is a major component of the growth-cone membrane preparation from the developing rat brain (Fig. 1).

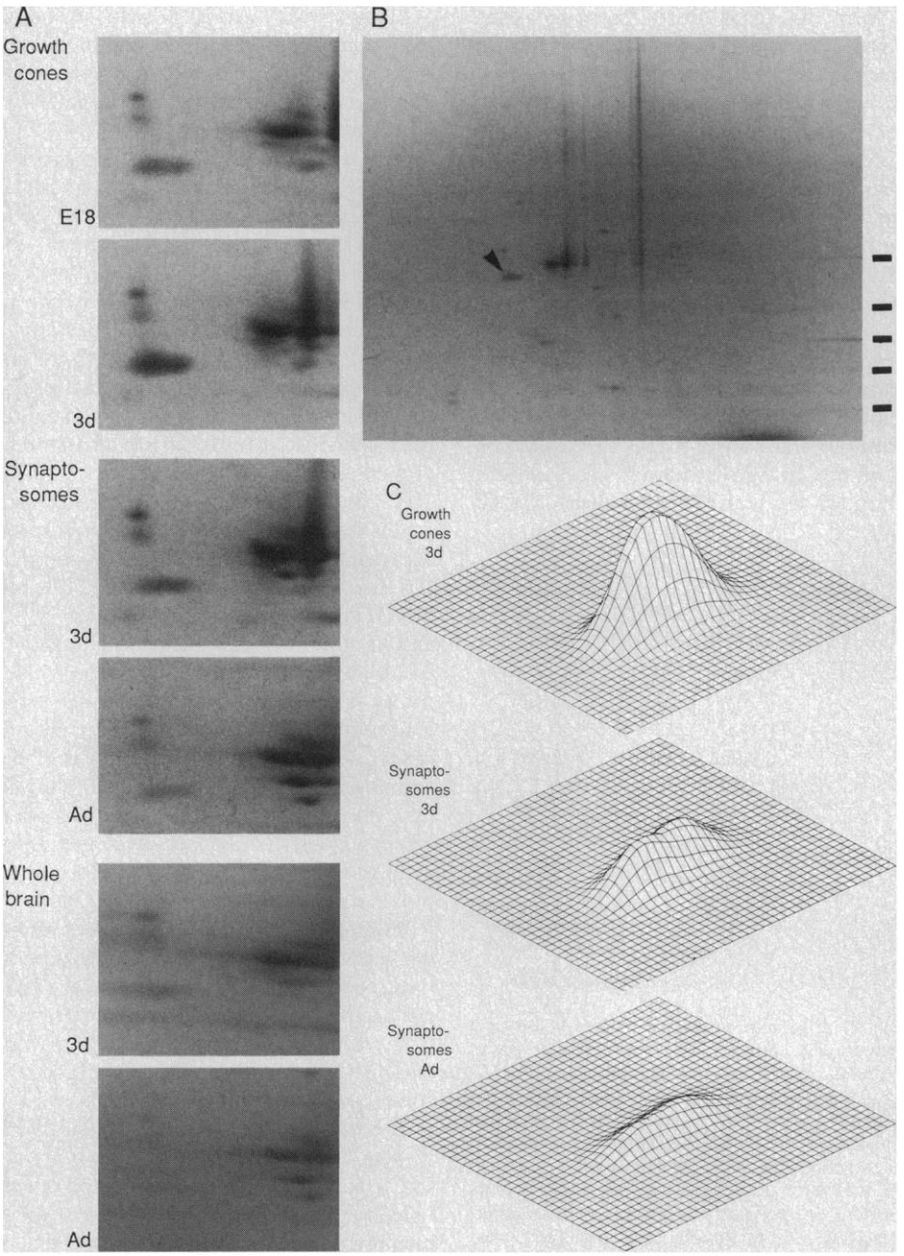

Fig. 2. Relative abundance of GAP-43 in growing and mature axon terminals. (A) Vignettes of Coomassie-stained gels showing the gel region containing GAP-43 from membrane fractions of growth-cone preparations, synaptosomes, and whole brains of fetal (E18), neonatal (3d), and adult (Ad) rats. Equal amounts of total protein were loaded onto each gel. (B) A whole Coomassie-stained gel of membrane proteins of a growth-cone fraction from 3-day-old rat brain shows that GAP-43 (arrow) is one of the most abundant proteins in the fraction. (C) Computer-directed 2-D densitometric analyses of the GAP-43 spot. Growth cones were isolated from the brains of postnatal rats (3 days old). Synaptosomes were isolated from 3-day-old and adult rat brains as described by Carlin *et al.* (*32*), and membrane fractions from these preparations were isolated as described for the growth-cone preparations. A total of 120 μg of protein was applied to each gel. Two-dimensional electrophoresis was carried out as described in the text, except that ampholytes were used at *p*H 4 to 6 instead of at *p*H 5 to 7. Gels were fixed and stained with Coomassie blue, dried on clear cellophane and scanned with a computer-based 2-D densitometer. The absolute amount of GAP-43 on each gel was estimated by comparison of GAP-43 staining with the staining of known amounts of molecular weight marker proteins.

Coomassie-stained gels show that, as a proportion of total protein, GAP-43 is more abundant in growth-cone membranes and in immature synaptic membranes than in a crude particulate fraction from 3-day rat brain (Fig. 2A). The distribution of GAP-43 illustrated in Fig. 2A is typical of 11 growth-cone and synaptosomal fractionations carried out with separate litters (5 to 11 pups per litter; growth-cone fractions were isolated from 18-day fetal brains on only two occasions). Densitometric analysis of one of these preparations indicated approximately six times as much GAP-43 in the growth-cone preparation as in a crude membrane fraction from the neonatal brain. GAP-43 is among the most abundant proteins detectable in growth-cone membranes by silver or Coomassie staining (Figs. 1 and 2B). When 120 μg of protein from growth-cone membranes is applied to a gel, the extent of Coomassie staining of the GAP-43 spot is consistent with the presence of at least 1 to 2 μg of protein, which suggests that GAP-43 constitutes more than 1% of the total growth-cone membrane protein.

Immunohistochemical localization of GAP-43 is also consistent with a specific association of the protein with growth cones in the developing brain. We have localized GAP-43 in light microscopic sections of neonatal rat brain. In early postnatal development, GAP-43 immunoreactivity is localized to very fine irregular profiles in a number of CNS structures, including the retinal recipient layers of the superior colliculus and the molecular layer of the dentate gyrus (Fig. 3A). In the dentate gyrus, GAP-43 immunoreactivity is pronounced at post-

natal day 10, which is also a time of prolific ingrowth of axons and synaptogenesis in this region (*19*). Little immunoreactivity could be detected in control sections reacted with antiserum that had been absorbed with purified GAP-43 (Fig. 3B). The presence of GAP-43 specifically within neuropil areas undergoing axon ingrowth and synapse formation is consistent with the biochemical evidence that the protein is predominantly associated with growth cones and immature synaptic endings.

GAP-43 can be detected in 2-D gels of membrane proteins from adult rat brains, although less is synthesized than in neonatal brains (*9*). The small amount of GAP-43 in adult brains appears to be concentrated in the synaptic membranes of mature axon terminals (Fig. 2A). We used a computer-based 2-D densitometric analysis (Microscan 1000, Technology Resources, Nashville, TN) to measure the abundance of GAP-43, relative to total protein, in growth-cone and synaptic membrane fractions from 3-day brains and in synaptic membranes from adult brains. Growth-cone membranes from 3-day-old rats contained 4.5 ± 1.2 (SEM) times as much GAP-43 per unit of total protein as synaptic membranes from 3-day brain (n = 3) and 12 ± 5 times as much as from adult synaptic membranes (n = 3), which suggests that the transition from the growth cone to the immature synaptic terminal is accompanied by a decrease in GAP-43 abundance that continues as synapses mature.

Quantitative comparisons of GAP-43 must be corrected to account for differences in protein and membrane content between growth cones and synaptosomes. Growth-cone membranes have less protein per unit of membrane [probably an amount similar to the protein content of myelin (*20*), at least 25% protein by weight] than synaptosomes [approximately 55% protein (*21*)]. However, growth cones are much larger than synaptic terminals and typically contain extensive internal membranes (*14, 20*). Isolated growth cones, for example, have approximately twice the mean diameter of isolated synaptosomes (*20*), which indicates a surface area four times as large. These considerations suggest that the concentration of GAP-43 per unit membrane is approximately six times as great in growth cones as in mature synaptic terminals, while the total amount of GAP-43 in a typical growth cone is on the order of 20 times as great as in a typical synaptic terminal.

The consistent pattern of GAP-43 induction during axon regeneration and development (*1–9*) and the abundance of GAP-43 in growth-cone membranes suggests that it may participate directly in some aspect or aspects of axonal growth. The presence of GAP-43 in mature synaptic terminals raises the possibility that it may participate in synaptic functions as well. We show elsewhere (*9*) that GAP-43 is electrophoretically identical to and antigenically similar to B-50, a preferred substrate for protein kinase C in synaptic terminals (*22, 23*). More phosphorylation of an electrophoretically similar protein by an endogenous kinase occurs in growth cones than in mature synaptosomes (*9, 24, 25*). That GAP-43 can often be resolved into two overlapping spots on 2-D gels (Fig. 2C) may reflect different states of phosphorylation of the protein. These results are interesting in light of the association of protein kinase C activation with stimulation of secretion, including neurotransmitter release at synaptic terminals (*26*), and with some steps in the regulation of cellular growth and transformation (*27*).

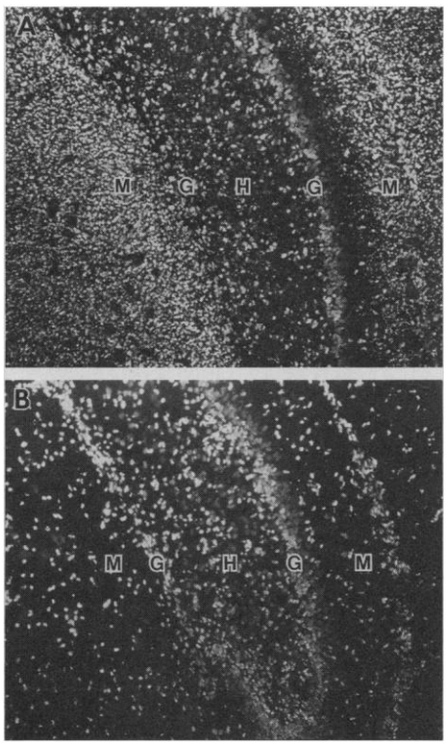

Fig. 3. Light microscopic immunohistochemical localization of GAP-43 in neonatal rat hippocampal formation (×100). A specific rabbit antiserum was made against rat GAP-43 isolated from preparative 2-D gels and used to localize GAP-43 in frozen sections of neonatal rat brain. Unfixed sections were cut at 10 μm in a cryostat and precipitation-fixed in 2:1 chloroform:methanol. Sections were then incubated in a 1:100 solution of H_2O_2 and methanol to block endogenous peroxidase activity. GAP-43 immunoreactivity was visualized by using standard peroxidase-anti-peroxidase methods (*33*), with 3,3′-diaminobenzidine used as the chromagen. The primary antiserum dilution was 1:500. These are dark-field photomicrographs of the dentate gyrus of a 10-day-old rat pup. (A) GAP-43 immunoreactivity is specifically localized to the molecular (M) or neuropil layers of the dentate gyrus, which are undergoing pronounced synaptogenesis at this age. No significant immunoreactivity is present in the granular (G) layers or hilus (H) of the dentate gyrus. (B) Adjacent control sections, which were reacted with antiserum to GAP-43 that had been absorbed with purified GAP-43 protein, show no immunoreactivity. Cell bodies in the granular layer are visible under dark-field in both experimental and control sections because of counterstaining of the sections with cresyl violet.

If GAP-43 participates in axon growth, GAP-43 expression could be an important site for the control of nerve regeneration after injury to the adult nervous system. When mature axons are severed, the GAP-43 concentrated in axon terminals must be lost as the distal portions of the axons degenerate. Because GAP-43 synthesis declines during maturation of the CNS (*4–6, 9*), injured neurons in adults might require increased GAP-43 synthesis in order to supply the amount of GAP-43 found in functional growth cones. Failure to increase GAP-43 synthesis after injury seems to distinguish abortive from successful regeneration (*1–6*). Injured axons in the mature mammalian CNS form terminal expansions resembling growth cones morphologically, but they fail to elongate (*28, 29*). We suggest that these "growth cones" are deficient in at least one major protein, GAP-43, and that this deficiency could limit the ability of the injured axons to regenerate.

Note added in proof: Meiri *et al.* (*30*) also have recently reported that GAP-43 is a major component of growth-cone membranes isolated from neonatal rat brains and have localized the protein to growth cones and elongating neurites of neurons in vitro.

REFERENCES AND NOTES

1. J. H. P. Skene and M. Willard, *J. Cell Biol.* **89**, 86 (1981).
2. L. I. Benowitz, V. E. Shashoua, M. G. Yoon, *J. Neurosci.* **1**, 301 (1981).
3. L. I. Benowitz and E. R. Lewis, *ibid.* **3**, 2153 (1983).
4. A. M. Heacock and B. W. Agranoff, *Neurochem. Res.* **7**, 771 (1982).
5. J. H. P. Skene and M. Willard, *J. Cell Biol.* **89**, 96 (1981).
6. K. Kalil and J. H. P. Skene, *J. Neurosci.*, in press.
7. S. Bock, G. J. Snipes, J. J. Norden, J. A. Freeman, *Soc. Neurosci. Abstr.* **10**, 1030 (1984).
8. J. A. Freeman *et al.*, *Exp. Brain Res.* (*Suppl.* 13), 34 (1986).
9. R. D. Jacobson, I. Virag, J. H. P. Skene, *J. Neurosci.* **6**, 1843 (1986).
10. S. C. Landis, *Annu. Rev. Physiol.* **45**, 567 (1983).
11. P. C. Letourneau, *Dev. Biol.* **44**, 92 (1975).
12. D. Bray and M. B. Bunge, *Ciba Found. Symp.* **14**, 195 (1973).
13. J. H. P. Skene and M. Willard, *J. Neurosci.* **1**, 419 (1981).
14. K. H. Pfenninger, L. Ellis, M. P. Johnson, L. B. Freidman, S. Somlo, *Cell* **35**, 573 (1983).
15. P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007 (1975).
16. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
17. G. Fairbanks, T. L. Steck, D. F. H. Wallach, *Biochemistry* **10**, 2606 (1971).
18. J. H. Morrisey, *Anal. Biochem.* **117**, 307 (1981).
19. W. M. Cowan, B. B. Stanfield, K. Kishi, in *Neural Development: Current Topics in Developmental Biology*, R. K. Hunt, Ed. (Academic Press, New York, 1980), vol. 15, pp. 103–157.

20. P. R. Gordon-Weeks and R. O. Lockerbie, *Neuroscience* **13**, 119 (1984).
21. C. Cotman, M. L. Blank, A. Moehl, F. Snyder, *Biochemistry* **8**, 4606 (1969).
22. V. J. Aloyo, H. Zweirs, W. H. Gispen, *J. Neurochem.* **41**, 649 (1983).
23. A. B. Oestreicher *et al.*, *ibid.*, p. 331.
24. F. Katz, L. Ellis, K. H. Pfenninger, *J. Neurosci.* **5**, 1402 (1985).
25. P. N. E. deGraan *et al.*, *Neurosci. Lett.* **61**, 235 (1985).
26. Y. Nishizuka, *Science* **225**, 1365 (1984).
27. W. L. Farrar, T. P. Thomas, W. B. Anderson, *Nature (London)* **315**, 235 (1985).
28. S. Ramón y Cajal, *Degeneration and Regeneration of the Nervous System* (Oxford Univ. Press, London, 1928), pp. 693–702.
29. C. Clemente, *Int. Rev. Neurobiol.* **6**, 257 (1964).
30. K. F. Meiri, K. H. Pfenninger, M. B. Willard, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3537 (1986).
31. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
32. R. K. Carlin, D. J. Grab, R. S. Cohen, P. Siekevitz, *J. Cell Biol.* **86**, 831 (1980).
33. L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, 1979).
34. We thank S. Bock, I. Virag, and M. Siegel for technical assistance. Supported by NIH grants EY01117 and NS18103 to J.A.F., EY03718 to J.J.N., and NS20178 to J.H.P.S.; by the Isabelle Niemela Fund; and by a Searle Scholars Award to J.H.P.S. R.D.J. was supported by a National Research Service Award.

10 October 1985; accepted 30 May 1986

Chromosome Y–Specific DNA Is Transferred to the Short Arm of X Chromosome in Human XX Males

MEA ANDERSSON, DAVID C. PAGE, ALBERT DE LA CHAPELLE*

Y-chromosomal DNA is present in the genomes of most human XX males. In these cases, maleness is probably due to the presence of the Y-encoded testis-determining factor (TDF). By means of in situ hybridization of a probe (pDP105) detecting Y-specific DNA to metaphases from three XX males, it was demonstrated that the Y DNA is located on the tip of the short arm of an X chromosome. This finding supports the hypothesis that XX maleness is frequently the result of transfer of Y DNA, including TDF, to a paternally derived X chromosome.

IN MAMMALS, SEX IS DETERMINED BY the presence or absence of the Y chromosome; males have a Y chromosome while females do not. One or more genes on the Y chromosome induces the undifferentiated gonad to become a testis; subsequent steps in sex differentiation are the result of the action of hormones. The gene(s) responsible for testicular determination has been termed the "testis-determining factor" (TDF) (*1*). The biochemical nature of TDF is unknown, but the gene has been localized to the short arm of the human Y chromosome (*2–3*).

Testes occasionally occur in the apparent absence of a Y chromosome. "XX males" are sterile men with small testes and an otherwise near-normal phenotype (*4*). Testicular differentiation in XX males might occur because of the presence of TDF in their genomes. To account for the acquisition of TDF and the anomalous inheritance of the X-linked blood group Xg in many XX males, it has been suggested that an aberrant X-Y interchange occurred during meiosis in the fathers (*5*).

The results of recent studies support the X-Y interchange hypothesis. As determined with X-linked restriction-fragment length polymorphisms, most if not all XX males indeed have one maternally derived and one paternally derived X chromosome (*6*). Direct evidence of X-Y exchange was provided by an XX male who expressed his father's allele for 12E7, a Y-linked marker, but failed to express his father's allele for Xg (*7*). When the genomes of 19 XX males were tested for the presence of 23 Y-specific restriction fragments, 12 of the males were found to have one or more of these DNA segments, while seven had none (*3, 8*). The TDF gene is probably close to these anonymous Y-specific DNA sequences present in the majority of XX males. However, the chromosomal location of the Y-derived DNA in those XX males is not known. The in situ hybridization experiments reported here were designed to answer this question.

The hybridization probe we used is pDP105, which detects a Y chromosome–specific family of DNA sequences (*9*). By the study of XX males, XYp− females, and XYq− males, it has been determined that most sequences homologous to pDP105 are found on Yp (and are present in some XX males), while other homologous sequences are found on Yq (*10*). There is no hybridization to female DNA at moderate stringencies.

For this study, we selected three XX males (LGL105, LGL115, and WHT950) (*11*) whose genomic DNA hybridized with pDP105 in Southern blotting experiments. To substantiate the normal chromosomal localization of pDP105 sequences, we also studied two normal 46,XY males.

Probe pDP105 was hybridized to meta-

Table 1. Grain counts after in situ hybridization of probe pDP105 to metaphase chromosomes from two normal 46,XY males and three 46,XX males. For the normal males and XX male LGL115, the metaphases were obtained from phytohemagglutinin-stimulated 3-day cultures of whole blood. For XX males LGL105 and WHT950, the metaphases were from Epstein Barr virus–transformed lymphoblastoid cell cultures. The metaphases were spread on microscope slides and hybridized with nick-translated probe DNA according to standard techniques (*16*). The air-dried chromosome preparations were denatured in 70% formamide, 0.3*M* NaCl, and 0.03*M* sodium citrate for 2 minutes at 70°C and hybridized in 50% formamide, 0.3*M* NaCl, 0.03*M* sodium citrate, and 10% dextran sulfate for 12 hours at 40°C with the ^{3}H-labeled probe (specific activity 9×10^{-6} count/min per microgram of DNA) at a concentration of 30 to 40 ng/ml. After hybridization, the slides were rinsed in 50% formamide, 0.3*M* NaCl, and 0.03*M* sodium citrate at 39°C. The slides were coated with Kodak NTB emulsion, developed after 5 to 14 days of exposure and stained with 0.25% Wright's stain (Gurr, BDH Chemicals Ltd. Poole, England). The metaphases were photographed and the analysis of grain distribution was made on the photographic print. In an alternative method, the slides were first stained with quinacrine hydrochloride (*17*) and suitable cells photographed. The slides were then rinsed in water and stained with Wright's stain. The distribution of the grains was determined by marking the localization of the grains as seen in the microscope on the photomicrographs showing Q-banding. Clusters of grains were counted as one grain.

Individual	Number of mitoses	Number of grains			Number of cells with at least one grain	
		Total	On Y (%)	On Xp22 (%)	On Y (%)	On Xp22 (%)
Normal male 1	28	174	33 (19)	2 (1)	23 (82)	2 (7)
Normal male 2	28	200	36 (18)	2 (1)	20 (71)	2 (7)
XX male LGL105	31	164		14 (9)		13 (42)
XX male LGL115	28	110		24 (22)		19 (68)
XX male WHT950	52	224		37 (17)		34 (65)

M. Andersson and A. de la Chapelle, Department of Medical Genetics, University of Helsinki, 00290 Helsinki, Finland.
D. C. Page, The Whitehead Institute for Biomedical Research, Cambridge, MA 02142.

*To whom correspondence should be addressed.