Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3209–3212, May 1986
Biochemistry

Activation of rat c-*raf* during transfection of hepatocellular carcinoma DNA

(DNA transfection/transforming gene/chemical carcinogen)

FUYUKI ISHIKAWA*, FUMIMARO TAKAKU†, KENSHI HAYASHI*, MINAKO NAGAO*, AND TAKASHI SUGIMURA*

*National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan; and †The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7-chome, Bunkyo-ku, Tokyo 104, Japan

Contributed by Takashi Sugimura, January 6, 1986

ABSTRACT **Rat c-*raf* was found to be activated in a transformant obtained with DNA of a hepatocellular carcinoma induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline. This activated c-*raf* was cloned in a cosmid vector and actively transforming clones were obtained. Comparison of the restriction maps of this activated c-*raf* and cloned normal rat c-*raf* revealed a recombination in the 5′-terminal region of the activated form of this gene. The recombined DNA was shown to be actively transcribed and possibly to form a fused mRNA with c-*raf*, which is slightly smaller than normal c-raf mRNA. Since this recombination was not detected in the original tumor by Southern blot analysis, it presumably occurred during transfection.**

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a potent mutagen present in broiled sardines (1) and cooked beef (2, 3), which was shown to be carcinogenic in mice (4) and rats (5). Thus, much attention has been paid to its possible involvement in human carcinogenesis (6).

To obtain information on the molecular mechanism of carcinogenesis by IQ, we used the method of NIH 3T3 cell transfection assay to study the activated oncogenes in seven rat tumors induced by IQ. We identified the transforming gene as activated rat Ha-*ras-1* in a transformant induced by one hepatocellular carcinoma, IQ4 (7) and also found activated rat c-*raf* in a transformant induced by another hepatocellular carcinoma, IQ7 (8).

The *raf* oncogene was first identified by Rapp *et al.* (9) as an oncogene of the murine transforming retrovirus 3611-MSV, which induces fibrosarcomas in newborn mice. The c-*raf* is the proto-oncogene of this v-*raf*. Human c-*raf-1* was cloned and its putative exons were sequenced (10). Results showed that this gene has at least 11 exons and extends over 20 kilobases (kb) in the human genome.

There are recent reports of activation of c-*raf* in a human gastric cancer and a glioblastoma line (11, 12). However, little is known about the genetic change responsible for the transforming activity of the activated c-*raf* or v-*raf*. Activated Ha-, Ki-, and N-*ras* genes in both human and experimental animal cancers were found to have a single point mutation (13–15). In contrast, here we report activation of c-*raf* by the grosser genetic change of recombination. We also show that, in this case, activation of c-*raf* occurred during transfection.

MATERIALS AND METHODS

Tumor. A hepatocellular carcinoma, IQ7, that had been induced in a male Fischer 344 rat by diet containing IQ (300 ppm) was used (5).

Cells. NIH 3T3 cells were maintained at low cell density in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. A clone with a low incidence of spontaneous transformants was selected and used for transfection assays. The transformed cells were maintained in DMEM with 5% calf serum.

DNA Transfection. High molecular weight DNAs were prepared from solid tumors and cultured cells as described (16). They were transfected into NIH 3T3 cells by the calcium phosphate precipitation method (17). For transfection of cloned cosmids, DNA samples of 0.02–12.5 μg were supplemented with carrier salmon sperm DNA to obtain samples containing a constant amount of DNA. Transformed foci were obtained after 14–21 days.

G418 Selection. G418 (18) was purchased from GIBCO. Cells were cultured in DMEM with G418 (400 μg/ml) and 10% calf serum. Typically, sensitive cells ceased to grow and became detached from the plates in 3 days. Resistance was judged after culture for 7 days.

Southern Blot Hybridization. Restriction endonucleases were purchased from Takara Shuzo (Kyoto, Japan) or Boehringer Mannheim and were used according to the manufacturers' instructions. DNAs were digested with restriction endonucleases, subjected to agarose gel electrophoresis, and transferred to nitrocellulose membrane filters by the method of Southern (19). The filters were hybridized in solution containing 50% formamide, 0.65 M NaCl, 0.1 M sodium Pipes (pH 6.8), 10% dextran sulfate, 5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% $NaDodSO_4$, 5 mM EDTA, and salmon sperm DNA (100 μg/ml) at 42°C with nick-translated probes for 18 hr, and then washed successively four times each with 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.2% sodium pyrophosphate/0.1% $NaDodSO_4$ for 20-min periods at 50°C.

RNA Blot Hybridization. Total RNA and poly(A)$^+$ RNA were prepared as described (20). A sample of 2 μg of poly(A)$^+$ RNA was denatured, subjected to electrophoresis on 0.9% agarose/formalin gel, and blotted onto nitrocellulose membrane filters. The hybridization and washing conditions were the same as for Southern blot hybridization.

Probes. The *Xho* I/*Bst*EII fragment (v-*raf* XB) of cloned v-*raf* (21) was used as a v-*raf*-specific probe. The *Bgl* I/*Hpa* I fragment from the 5′ end of the v-*raf* gene was used to determine the 5′–3′ orientation of the cloned gene.

Molecular Cloning. A genomic library of a secondary transformant was constructed in a cosmid vector, pCV108, as described (22). pCV108 was obtained from Y.-F. Lau. Briefly, the DNA was partially digested with *Mbo* I, size-fractionated in a sucrose density gradient, and ligated to a dephosphorylated *Bam*HI digest of pCV108. The ligated DNA was packaged *in vitro* and transduced into ED8767. The transformed bacteria were selected with ampicillin and the library was screened as described (23) with v-*raf* probe. A

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase(s); IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

genomic library of normal Fischer 344 rat was constructed in EMBL3A (24). High molecular weight DNA was prepared from normal Fischer 344 rat liver, partially digested with *Mbo* I, size-fractionated in a sucrose density gradient, dephosphorylated with calf intestinal phosphatase, and ligated to *Bam*HI/*Eco*RI double-digested EMBL3A. The ligated DNA was packaged *in vitro* and transduced into ED8654. The library was amplified and screened as described (25) with v-*raf* probe. Several DNA fragments were subcloned in pGEM2 (Promega Biotec, Madison, WI) with or without *Eco*RI linker (Collaborative Research, Waltham, MA).

RESULTS

Molecular Cloning of Activated and Normal Rat c-*raf*. On NIH 3T3 transfection assay, 60 μg of DNA of the original tumor IQ7 gave two primary transformants. Both were shown to contain rat-specific repetitive sequences, but only one clone, IQ7-2, had the rat c-*raf* sequence. DNA of IQ7-2 reproducibly yielded secondary and tertiary transformants in subsequent cycles of transfection assay, and these transformants were also shown to have the rat c-*raf* sequence (8). High molecular weight DNA was prepared from one of the secondary transformants and partially digested with *Mbo* I. The digest was size-fractionated in a sucrose density gradient and the fractions of 35–45 kb were pooled. This sample was ligated to the *Bam*HI site of pCV108, packaged *in vitro*, and transduced into ED8767. About 0.25 μg of the insert DNA sample gave 1×10^5 colonies, 12 of which were shown to contain the c-*raf* sequence in tests with v-*raf* as a probe. Seven clones were examined further. By comparison of their restriction patterns with that of cloned normal rat c-*raf* (see below), one of the seven clones was concluded to be derived from the NIH 3T3 mouse c-*raf* sequence. The abundance of clones of rat c-*raf* relative to that of mouse c-*raf* was thought to be due to amplification of rat c-*raf* in the secondary transformant.

For construction of a normal rat genomic library, high molecular weight DNA was prepared from normal F344 rat liver, partially digested with *Mbo* I, and size-fractionated in a sucrose density gradient. The ≈15-kb fragment was dephosphorylated with calf intestinal phosphatase and ligated to *Eco*RI and *Bam*HI double-digested EMBL3A. About 0.25 μg of the insert DNA gave 1×10^6 plaques, seven clones of which were identified with v-*raf* probe as containing c-*raf*.

Transforming Activities of Cosmid Clones. To determine which part of the activated *raf* gene is essential for the transforming activity, we transfected the cosmid clones into NIH 3T3 cells with carrier salmon sperm DNA (Table 1). Samples of up to 12.5 μg of DNAs of clones pRF1, -10, -11, and vector pCV108 gave no transformed foci. In contrast, pRF7 and -9 showed prominent transforming activities of 300–400 focus-forming units per μg of DNA. Similar results were obtained in another independent experiment. As the cosmid vector pCV108 has a selectable marker sequence derived from pSV2neo, we examined several transformants induced by pRF7 and -9 for G418 resistance. All clones examined were resistant to G418 (data not shown). This supports the conclusion that the transformation is due to incorporation of the cosmids. These results indicated that pRF7 and -9 contained the whole activated *raf* gene sequence.

Table 1. Transforming activities of cloned cosmids

Clone	DNA,* μg	No. of foci/no. of recipient cultures
pCV108	12.5	0/3
pRF1	12.5	0/3
pRF7	0.5	159/3
pRF9	0.5	204/3
pRF10	12.5	0/3
pRF11	12.5	0/3

*Salmon sperm DNA was added to cosmid DNA in a total of 60 μg and was transfected into cells in three dishes.

Restriction Maps of Normal and Activated Rat c-*raf*. Fig. 1 shows the restriction maps of normal and activated c-*raf*. The 5′–3′ orientation was determined by hybridization with a 5′-half fragment of v-*raf*. Comparison of the maps of normal and activated c-*raf* showed that the 5′ ends of v-*raf* homologous regions have different restriction patterns. This indicates that the activated c-*raf* has a recombination in its 5′-terminal region.

As shown above, the cosmid clones pRF7 and -9, which have almost the same region of c-*raf* as inserts, had transforming activity, whereas clones pRF1, -10, and -11 did not. Interestingly, pRF7 and -9 differ from pRF1 in having a relatively short (≈4 kb) region located upstream of the recombination point, which is not present in pRF1. The fact that pRF1 had no transforming activity suggests that there is an essential element in this 4-kb stretch and hence that the recombination is responsible for the transforming activity.

Position of the Recombination Point in Activated c-*raf*. To locate the recombination point, we constructed several subclones from normal and activated c-*raf*. Subclones pN1 and pR2 contained 6.5- and 5.7-kb *Hin*dIII fragments of normal and activated c-*raf*, respectively (Fig. 1). The recombination of activated c-*raf* occurred within the region of the pR2 insert, and pN1 is its normal counterpart. Comparison of their restriction maps showed differences in the 5′-terminal regions (Fig. 2). Again, we subcloned the 5′ end *Hin*dIII/*Ava* II fragments from pN1 and pR2, which we named pNAH and

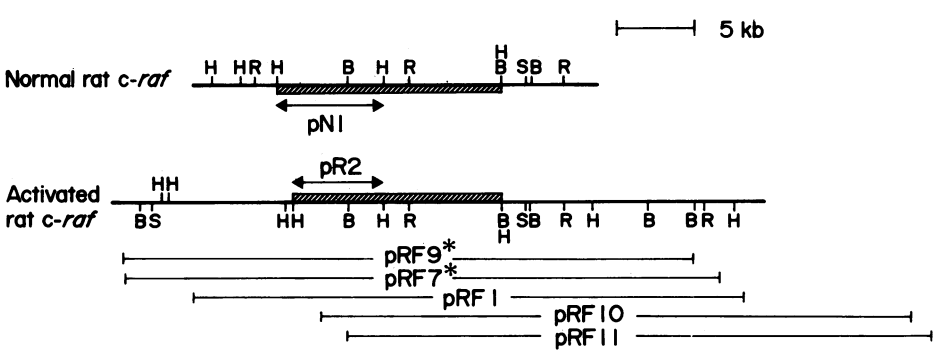

FIG. 1. Restriction maps of normal and activated rat c-*raf*. Hatched boxes indicate fragments having homology with v-*raf* XB. The positions of cosmid clones and subclones pN1 and pR2 are shown. The asterisks on pRF7 and -9 indicate that they have active transforming activity. The following restriction enzymes were used: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal* I.

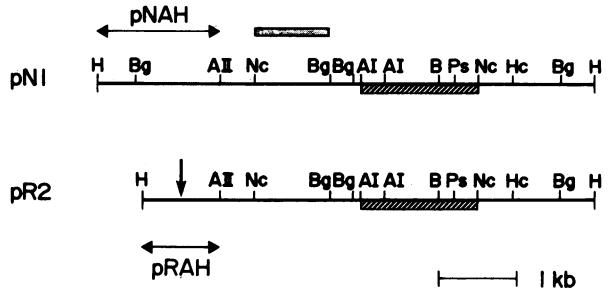

FIG. 2. Restriction maps of subclones pN1 and pR2. Hatched boxes indicate fragments having homology with v-*raf* XB. Shaded box indicates the *Nco* I/*Bgl* I fragment used as a probe. The positions of subclones pNAH and pRAH are shown. The arrow indicates the approximate position of the recombination site. The following restriction enzymes were used: AI, *Ava* I; AII, *Ava* II; B, *Bam*HI; Bg, *Bgl* I; H, *Hin*dIII; Hc, *Hin*cII; Nc, *Nco* I; Ps, *Pst* I. *Ava* II has other restriction sites besides those indicated.

pRAH, respectively. Partial digestions with the 4-base restriction enzymes *Alu* I and *Hae* III indicated that the recombination point was ≈500 base pairs upstream from the *Ava* II site (data not shown).

v-*raf* XB, the probe used, hybridizes with exons 9–16 of cloned human c-*raf* (ref. 10; numbering of exons is according to ref. 26). This probe hybridized with *Ava* I/*Nco* I fragments of pN1 and pR2 (Fig. 2). We did not detect hybridization between v-*raf* XB and the fragments within 10 kb upstream of this *Ava* I site. So, the homology between v-*raf* and rat c-*raf* can be assumed to terminate at this *Ava* I/*Nco* I fragment. We also used the 1-kb-long *Nco* I/*Bgl* I fragment of pN1, which is located ≈1 kb downstream of the recombination point (Fig. 2) as a probe in Southern blot hybridization. In human placenta DNA, this probe detected a 3.3-kb fragment of a *Hin*dIII digest and a 2.9-kb fragment of an *Xba* I digest (data not shown). The sizes of these fragments correspond to those containing exons 7–9 of human c-*raf-1* (10). So the *Nco* I/*Bgl* I fragment of pN1 was suggested to contain all or part of the sequences of exons 7–9. As the recombination point is ≈1 kb upstream of this fragment, we conclude that the recombination occurred in or around exons 7–9.

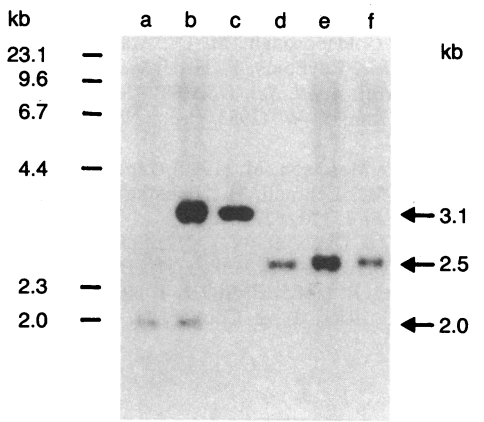

FIG. 3. Southern blot analysis of the hepatocellular carcinoma IQ7. Samples of 10 μg of DNAs were digested with *Bgl* I, subjected to electrophoresis in 0.9% agarose gel, and analyzed by blot hybridization with the 1-kb *Nco* I/*Bgl* I fragment of pN1 (Fig. 2) as a probe. Lanes: a, NIH 3T3; b, primary transformant IQ7-2; c, cosmid clone pRF7; d, normal rat c-*raf* cloned in EMBL3A; e, hepatocellular carcinoma IQ7; f, normal rat liver. The cosmid and λ clones were applied with salmon sperm DNA to give totals of 10 μg of DNAs.

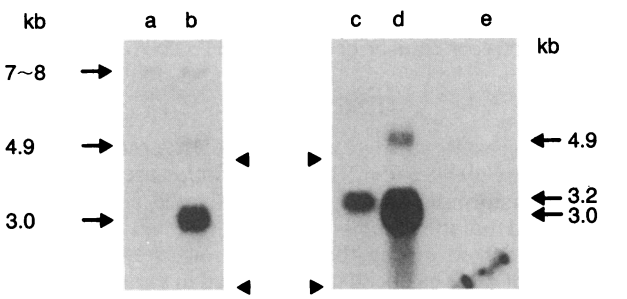

FIG. 4. RNA blot analysis of the primary transformant IQ7-2. Samples of 2 μg of poly(A)$^+$ RNAs were denatured, subjected to electrophoresis in 0.9% denaturing gel, and analyzed by blot hybridization with the insert of pRAH (lanes a and b) or v-*raf* XB (lanes c–e) as probe. Lanes: a and c, NIH 3T3; b and d, IQ7-2; e, normal rat liver. Triangles indicate the positions of 28S and 18S rRNA.

Absence of Recombination in the Original Tumor. We used the *Nco* I/*Bgl* I fragment (Fig. 2) as a probe to determine whether this recombination was present in DNA of the original tumor, IQ7. The results of Southern blot hybridization analysis of *Bgl* I digests with this probe are shown in Fig. 3. DNA from normal rat liver (lane f) and cloned normal rat c-*raf*, λNF6 (lane d), gave the same 2.5-kb band, whereas DNAs from the primary transformant (lane b) and cloned activated rat c-*raf*, pRF9 (lane c), gave the same 3.1-kb band. DNA from the original tumor, IQ7, gave exactly the same band as normal rat c-*raf* (lane e). Even on longer exposure of the film, no 3.1-kb fragment was detected in IQ7 (data not shown). These data strongly suggest that the recombination occurred during transfection.

Expression of Recombined DNA and c-*raf* in the Same mRNA. When we used the insert of pRAH as a probe in RNA blot hybridization, the RNA of NIH 3T3 cells gave a single band of 7–8 kb (Fig. 4, lane a). In the primary transformant IQ7-2, we found that in addition to this 7- to 8-kb mRNA, 4.9-kb and strongly hybridized 3.0-kb mRNAs were expressed (lane b). When the same filter was hybridized with the insert of pNAH, no hybridization was detected in either lane (data not shown). When the filter was hybridized with a v-*raf* probe, the primary transformant and NIH 3T3 cells gave a 3.2-kb band and the primary transformant also gave bands of 4.9- and 3.0-kb mRNA (lanes c, d). The RNA of normal Fischer 344 rat liver was also shown to contain a 3.2-kb transcript that hybridized with v-*raf* (lane e). The sizes, 4.9 kb and 3.0 kb, of mRNAs hybridized with v-*raf* were the same as those observed with pRAH insert as a probe. The inserts of pNAH and pRAH are equivalent fragments of normal and activated c-*raf*, respectively, and the latter contains the recombination region. The fact that pNAH shows no hybridization means that the insert of pNAH contains no exon or that expression of the exon is strongly suppressed. Hybridization with pRAH revealed that the DNA region homologous to that recombined to c-*raf* is expressed in NIH 3T3 cells and that this region is also actively transcribed in the primary transformant. However, as the 3.0- and 4.9-kb transcripts homologous to the pRAH insert were unique to the transformant and they were also detected with the v-*raf* probe, c-*raf* and the recombined region, the region of the pRAH insert, are very probably transcribed in the same mRNA in the transformant. Transcription of the activated c-*raf* begins upstream of the recombination point, reads through the junction, and may be terminated by a termination signal of the c-*raf*. The 4.9-kb transcript may be the precursor form of the 3.0-kb mRNA. The fact that the 7- to 8-kb mRNA was detected with pRAH in both NIH 3T3 cells and the primary transformant indicates

that the normal gene containing the region homologous to pRAH is actively transcribed in NIH 3T3 cells.

DISCUSSION

The present results strongly suggest that DNA recombination and qualitatively altered mRNA transcription are responsible for the transforming activity of the activated c-*raf*. Results also showed that the recombination that resulted in activation of c-*raf* occurred during the transfection assay. Recently, activation of c-*raf* has been demonstrated in various cancers, human stomach cancer (11), a glioblastoma line (12), and a pancreatic and a liver cancer induced by azaserine in rats.‡ It is very important to elucidate whether these c-*raf* genes were activated during development of cancer *in vivo* or during transfection *in vitro*.

Recently, *ret* was reported to be activated by recombination of tumor DNA in NIH 3T3 cells (27). This report together with the present results indicates that activation of oncogenes may not be infrequent during the process of transfection of DNA into NIH 3T3 cells. Thus, examination of the original tumor is important, at least in studies on oncogenes activated by rearrangement.

Bonner *et al.* (10) suggested that in activation of c-*raf* the amino acid difference between the exons of v-*raf* and human c-*raf-1* was not essential for the transforming activity of v-*raf*. We also detected a recombination of c-*raf* similar to that described here in a transformant induced by DNA of a human colon cancer (unpublished observations). So we consider that the recombination described here is a necessary event for the activation of c-*raf*.

Mölders *et al.* (26) reported that the Moloney leukemia virus long terminal repeat (LTR) insertion between exons 4 and 5, and a high level of expression of the hybrid transcript initiated from the LTR promoter can activate c-*raf*. Thus, deletion of 5′ exons of c-*raf* and formation of truncated or fusion protein seem to be crucially important for its activation.

Further analyses of transcripts and translation product of this activated c-*raf* should provide conclusive information on the c-*raf* activation during the transfection process and on the mechanisms of this activation.

As IQ is one of the environmental carcinogens, it is important to know the molecular mechanism of carcinogenesis by IQ. In a series of tumors induced by IQ, we identified activated rat Ha-*ras-1* in a transformant induced by one hepatocellular carcinoma, IQ4 (7). It is highly probable that this Ha-*ras* was also activated in the original tumor, IQ4, since there is no report that the activation of *ras* genes by point mutation occurred during transfection. We are now examining whether a point mutation of Ha-*ras-1* is present in the DNA of IQ4.

‡Zurlo, J., Silverman, J. A. & Yager, J. D. First Annual Meeting on Oncogenes, July 1985, Frederick, MD, p. 171 (abstr.).

We thank Dr. Y.-F. Lau for providing pCV108. This study was supported by a grant-in-aid from the Ministry of Health and Welfare for Comprehensive 10-Year Strategy for Cancer Control, Japan, and by grants from the Princess Takamatsu Cancer Research Fund and Hishinomi Cancer Research Fund. F.I. was an awardee of Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

1. Kasai, H., Nishimura, S., Wakabayashi, K., Nagao, M. & Sugimura, T. (1980) *Proc. Jpn. Acad.* **56B**, 382–384.
2. Felton, J. S., Knize, M. G., Wood, C., Wuebbles, B. J., Healy, S. K., Stuerner, D. H., Bjeldanes, L. F., Kimble, B. J. & Hatch, F. T. (1984) *Carcinogenesis* **5**, 95–102.
3. Hayatsu, H., Matsui, Y., Ohara, Y., Oka, T. & Hayatsu, T. (1983) *Gann* **74**, 472–482.
4. Ohgaki, H., Kusama, K., Matsukura, N., Morino, K., Hasegawa, H., Sato, S., Sugimura, T. & Takayama, S. (1984) *Carcinogenesis* **5**, 921–924.
5. Takayama, S., Nakatsuru, Y., Masuda, M., Ohgaki, H., Sato, S. & Sugimura, T. (1984) *Gann* **75**, 467–470.
6. Sugimura, T. (1985) *Mutat. Res.* **150**, 33–41.
7. Ishikawa, F., Takaku, F., Nagao, M., Ochiai, M., Hayashi, K., Takayama, S. & Sugimura, T. (1985) *Jpn. J. Cancer Res. (Gann)* **76**, 425–428.
8. Ishikawa, F., Takaku, F., Ochiai, M., Hayashi, K., Hirohashi, S., Terada, M., Takayama, S., Nagao, M. & Sugimura, T. (1985) *Biochem. Biophys. Res. Commun.* **132**, 186–192.
9. Rapp, U. R., Reynolds, F. H., Jr., & Stephenson, J. R. (1983) *J. Virol.* **45**, 914–924.
10. Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G. & Rapp, U. R. (1985) *Mol. Cell. Biol.* **5**, 1400–1407.
11. Shimizu, K., Nakatsu, Y., Sekiguchi, M., Hokamura, K., Tanaka, K., Terada, M. & Sugimura, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5641–5645.
12. Fukui, M., Yamamoto, T., Kawai, S., Maruo, K. & Toyoshima, K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5954–5958.
13. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Shang, E. H. (1982) *Nature (London)* **300**, 143–149.
14. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300**, 149–152.
15. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) *Nature (London)* **306**, 658–661.
16. Pellicer, A., Wigler, M., Axel, R. & Silverstein, S. (1978) *Cell* **14**, 133–141.
17. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. C. & Axel, R. (1977) *Cell* **11**, 223–232.
18. Davies, J. & Jimenez, A. (1980) *Am. J. Trop. Med. Hyg.* **29**, 1089–1092.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Feramisco, J. R., Smart, J. E., Burridge, K., Helfman, D. M. & Thomas, G. P. (1982) *J. Biol. Chem.* **257**, 11024–11031.
21. Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr., & Stephenson, J. R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4218–4222.
22. Lau, Y.-F. & Kan, Y. W. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5225–5229.
23. Hanahan, D. & Meselson, M. (1980) *Gene* **10**, 63–67.
24. Frischauf, A.-M., Lehrach, H., Poustka, A. & Murray, N. (1983) *J. Mol. Biol.* **170**, 827–842.
25. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
26. Mölders, H., Defesche, J., Müller, D., Bonner, T. I., Rapp, U. R. & Müller, R. (1985) *EMBO J.* **4**, 693–698.
27. Takahashi, M., Ritz, J. & Cooper, G. M. (1985) *Cell* **42**, 581–588.Z