MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3380–3385
0270-7306/87/103380-06$02.00/0

Vol. 7, No. 10

A Factor Present in Fetal Calf Serum Enhances Oncogene-Induced Transformation of Rodent Fibroblasts

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Received 23 March 1987/Accepted 22 June 1987

Our previous studies indicated that addition of the tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or teleocidin to Dulbecco modified Eagle medium supplemented with calf serum enhanced T24-induced focus formation in both the murine C3H 10T1/2 and rat 6 embryo fibroblast cell lines. In the present studies we have found that fetal calf serum (FCS) is more potent than 12-*O*-tetradecanoylphorbol-13-acetate in enhancing T24-induced focus formation, in terms of the number and size of the foci, in both C3H 10T1/2 and rat 6 cells. Time course studies indicate that FCS can exert this enhancing effect when it is added several days after the transfection with T24 DNA. In rat 6 cells, an 11-fold increase in T24-induced focus formation occurred when the transfected cultures were maintained for only 1 day in 5% FCS, starting 4 days after the transfection. Several known growth factors, including epidermal growth factor, transforming growth factors α and β, insulin, and platelet-derived growth factor, did not enhance T24-induced transformation in these cell systems. Fractionation studies indicate that the factor present in FCS has a molecular weight of about 1,300, is not lipid soluble, and is acid, base, and heat stable. These findings suggest that a factor(s) normally present in serum may enhance the emergence of tumor cells in vivo, by acting in concert with an activated oncogene, during the multistage carcinogenic process.

Carcinogenesis induced by various agents, including chemicals, radiation, and viruses, in rodents, humans, and cell culture systems, is usually a multistep process (see reference 17 for a review). We previously reported that the potent tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and teleocidin enhance the transformation of cultured rodent fibroblasts cell lines that had been transfected with the activated human oncogene c-Ha-*ras* T24 (11, 12). Similar findings have been obtained by Dotto et al. (5) for early-passage rat embryo fibroblast cultures. Evidence has also been obtained that in such systems, the tumor promoters do not act simply by enhancing the process of transfection per se (5, 11, 12). These findings suggest that during multistage carcinogenesis, tumor promoters might act by complementing the function of activated cellular oncogenes. We now report that a factor present in fetal calf serum (FCS) is particularly potent in enhancing expression of the transformed phenotype in murine and rat cells transfected with the activated c-Ha-*ras* oncogene.

MATERIALS AND METHODS

Materials. TPA was purchased from LC Service Corp., Woburn, Mass. Bovine transforming growth factors α and β (TGF-α and TGF-β) were gifts from Michael Sporn, National Cancer Institute, Bethesda, Md. Platelet-derived growth factor (PDGF) was provided by R. Lehrman, Roche Institute of Molecular Biology, Nutley, N.J. Mouse epidermal growth factor (EGF) and CR-ITS (premix of insulin, transferrin, and selenium) were obtained from Collaborative Research, Inc., Bedford, Mass. Insulin, transferrin, and selenium were from Sigma Chemical Co., St. Louis, Mo. G418 sulfate (geneticin) was obtained from GIBCO Laboratories, Grand Island, N.Y. Spectrapor dialysis tubing were obtained from Fisher Scientific, Co., Springfield, N.J. Gel filtration standards and Bio-Gel P10 were from Bio-Rad Laboratories, Richmond, Calif. Calf serum (CS) and FCS were obtained from Flow Laboratories, Inc., McLean, Va.

Cells and culture conditions. Rat 6 cells were subcloned from an immortalized F2408 embryo fibroblast cell line obtained from the laboratory of C. Basilico; they were originally derived by Freeman et al. (7). The C3H 10T1/2 C18 mouse embryo fibroblast cell line was originally established by Reznikoff et al. (16). Stock cultures of both cell types were grown in 90-mm dishes containing Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% CS. Cultures were maintained in a humidified incubator at 37°C under 5% CO_2 in air.

Plasmids. Plasmid pSV2-gpt contains an *Escherichia coli gpt* gene coding for xanthine-guanine phosphoribosyltransferase linked to the simian virus 40 origin of DNA replication and early promoter region (14). Plasmid pIBW (*tk-neo*) contains a bacterial *neo* gene that confers resistance to neomycin and kanamycin; this gene is under the transcriptional regulation of the promoter sequence derived from the herpes simplex virus thymidine kinase (*tk*) gene, constructed in the laboratory of R. Axel. Plasmid pT24 contains a 6.4-kilobase *Bam*HI sequence corresponding to the cellular coding sequence of the mutated human bladder cancer c-Ha-*ras* oncogene (9) and was obtained from M. Wigler. Plasmid pMuLV-myc contains the v-*myc* gene driven by the Moloney murine leukemia virus promoter, and plasmid pMc-myc 54 contains the murine c-*myc* cDNA sequence. The last two plasmids were obtained from M. Goldfarb.

Transfection procedure. The calcium phosphate-mediated DNA transfection procedure previously described by Bacchetti and Graham (1) and Goldfarb et al. (9) was used with minor modifications (12). To test the effect of FCS on T24-induced focus formation, 5% FCS was substituted for 5% CS, as indicated in each experiment. Fractions isolated from FCS and specific growth factors were added to growth medium containing 5% CS beginning 4 days after the trans-

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TABLE 1. Transfection frequencies obtained with pT24, pSV2-gpt, and ptkneo DNAs in rodent embryo fibroblasts in the presence of CS or FCS

Recipient cells	No. of colonies (foci)/5 × 10^5 recipient cells					
	gpt colonies		*neo* colonies		T24 foci	
	CS	FCS[a]	CS	FCS[a]	CS	FCS[a]
C3H 10T1/2						
Expt 1	204	440	ND[b]	ND	5	84
Expt 2	771	545	ND	ND	38	193
Rat 6						
Expt 1	ND	ND	1,289	1,286	5	52
Expt 2	ND	ND	1,686	1,700	3	99

[a] Cells were grown in the presence of 5% FCS beginning 1 day after transfection and continuing throughout the experiment.
[b] ND, Not done.

fection procedure and during each refeeding with fresh medium, unless indicated otherwise. Cells were fed every other day for the first week and twice a week for the remainder of the experiment. At 2 to 3 weeks after transfection, plates were stained with Giemsa and scored for transformed foci. In transfection studies with the *gpt* or *neo* gene, colonies were selected for drug resistance as previously described (12).

Preparation of cellular DNA and RNA and nucleic acid hybridizations. Cellular DNA samples were prepared by the method of Gattoni-Celli et al. (8). Total cellular RNA samples were prepared by the cesium chloride cushion method (4). A *Sac*I-*Sac*I 3.0-kilobase T24-specific fragment obtained from plasmid pT24 and an *Eco*RI-*Eco*RI 1.1-kilobase c-*myc* cDNA sequence derived from plasmid pMc-myc 54 were used for the Southern and Northern (RNA blot) hybridization analyses, respectively. These procedures were performed as previously described (8).

Fractionation of FCS. A Bio-Gel P10 (200/400 mesh) column (diameter, 1.5 cm; height, 65 cm) was packed and equilibrated with 0.01 M phosphate buffer (pH 7.0) at a flow rate of 5 ml/h at 4°C. FCS was dialyzed at 4°C against 10 volumes of double-distilled H_2O by using dialysis tubing with a 12,000- to 14,000-molecular-weight cutoff. The dialyzate was lyophilized and suspended in double-distilled H_2O to 1/20 its original volume. A small amount of insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. Portions of the supernatant fraction (1 to 5 ml) were then applied to the P10 column and eluted with 0.01 M phosphate buffer (pH 7.0). Fractions (2 ml) of the eluate were collected, monitored for A_{230}, A_{260}, and A_{280}, and pooled (see Fig. 5 legend). The pooled fractions were lyophilized, redissolved in double-distilled H_2O, sterile filtered, and assayed for their ability to enhance T24 focus formation in rat 6 cells.

RESULTS

Effects of FCS on the formation of T24-induced foci. To study possible synergistic interactions between factors present in FCS and an activated human c-H-*ras* oncogene (T24), C3H 10T1/2 or rat 6 cells were grown and then transfected with T24 DNA in Dulbecco modified Eagle medium supplemented with 10% CS as described previously (11). At 24 h later the transfected cells were replated in either Dulbecco modified Eagle medium–5% CS or Dulbecco modified Eagle medium–5% FCS. In parallel studies, cells were transfected with the *neo* or *gpt* gene instead of T24 and, on day 2, were replated in G418 or XAT medium (hypoxanthine, 15 μg/ml; aminopterin, 0.2 μg/ml; thymidine, 5 μg/ml; xanthine, 25 μg/ml; mycophenolic acid, 25 μg/ml), respectively, containing either 10% CS or 10% FCS. Growth in FCS enhanced T24-induced focus formation 5- to 17-fold in C3H 10T1/2 cells and 10- to 33-fold in rat 6 cells when compared with the results obtained with CS (Table 1). On the other hand, FCS did not enhance the number of Gpt^+ or Neo^+ colonies obtained. In these studies, and in those presented below, neither C3H 10T1/2 nor rat 6 cells displayed transformed foci when transfected with normal carrier DNA, in the presence of either CS or FCS. T24-induced foci also appeared earlier in cultures fed FCS rather than CS (6 days versus 10 to 12 days), and the foci seen in the FCS-treated culture were also much larger and appeared more transformed (Fig. 1) in both the C3H 10T1/2 and rat 6 cell cultures. In the rat 6 cell cultures, the foci seen in the presence of FCS were very dense and the cells were round and formed cell clumps (Fig. 1). The morphology of the transformed foci remained the same when the cultures were subsequently transferred from medium containing FCS to medium containing CS. In addition, when a number of clones were established from the C3H 10T1/2 and rat 6 transformed foci, these cells continued to display a transformed morphology whether grown in the presence of CS or FCS.

DNAs were extracted from a number of these transformed clones and analyzed for the presence of integrated copies of the T24 gene by Southern blot analysis. Figures 2a and b reveal the presence of T24-specific DNA sequences in all of the transformed foci examined. No consistent difference was seen between clones obtained from cultures grown in the presence of FCS or CS in terms of the number of integrated copies of T24 DNA sequences. Thus FCS does not appear to act by enhancing the integration of the T24 DNA. Consistent with this interpretation is the fact that FCS did not enhance the recovery of Neo^+ or Gpt^+ colonies (Table 1).

We also explored the effects of time and duration of FCS treatment on the enhancement of T24-induced foci. Studies with the C3H 10T1/2 cells indicated that maximum enhance-

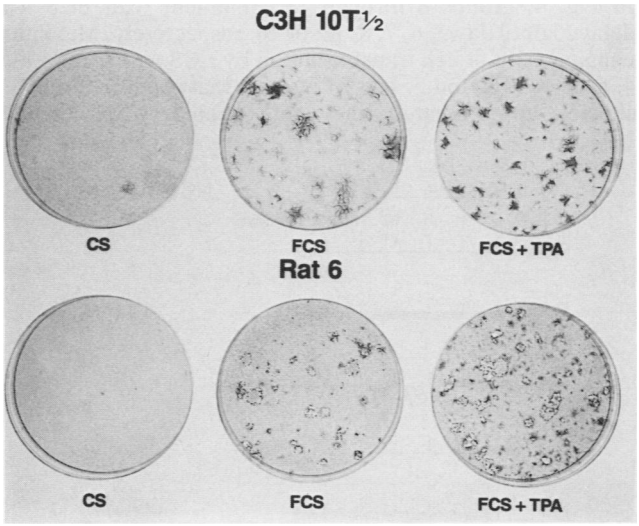

FIG. 1. Cell culture plates of C3H 10T1/2 and rat 6 cells stained with Giemsa 24 days after transfection with pT24 DNA. Following transfection, cells were grown in the presence of 5% CS, 5% FCS, or 5% CS plus 100 ng of TPA per ml. For additional details, see Materials and Methods.

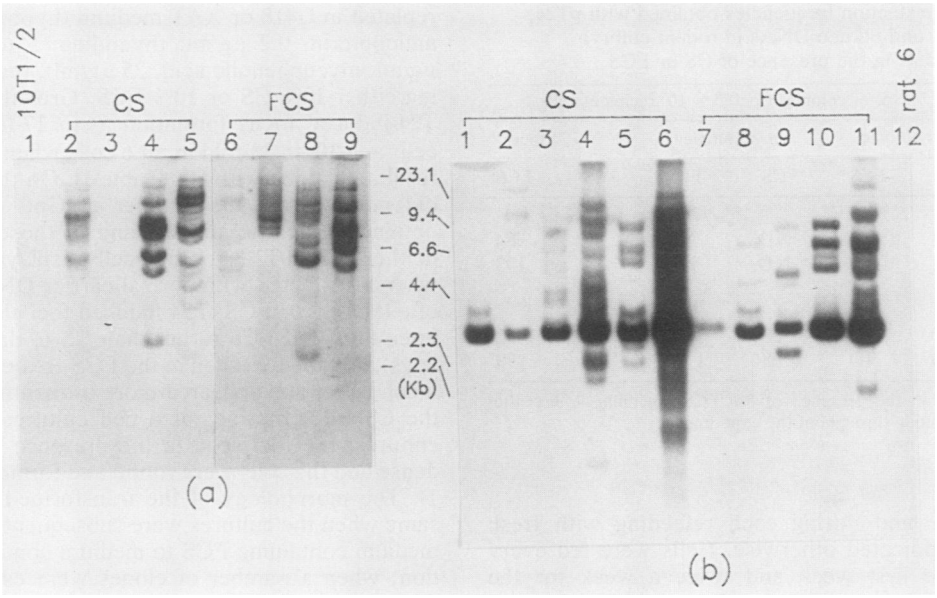

FIG. 2. Southern blot analyses of chromosomal DNAs extracted from T24 transformed colonies isolated from C3H 10T1/2 cells (a) and rat 6 cells (b). Lanes 1, normal 10T1/2 cells; lane 12, normal rat 6 cells; lanes 2 to 5 (panel a) and 1 to 6 (panel b), transformed colonies obtained from transfection studies done with CS; lanes 6 to 9 (panel a) and 7 to 11 (panel b), transformed colonies obtained from transfection studies done with FCS. DNAs were digested with *Eco*RI (panel a) or *Sac*I (panel b), electrophoresed, blotted, and hybridized with a ^{32}P-labeled probe to T24. For additional details, see Materials and Methods.

ment was obtained when the cells were grown in the presence of FCS beginning 1 day after the transfection procedure and throughout the remainder of the experiment (Fig. 3, group E). Additional treatment of the cells with FCS beginning 1 day before the transfection procedure did not yield additional foci (Fig. 3, group B). This was also the case with rat 6 cells (data not shown). More detailed time course studies were then performed with the rat 6 cells. In Fig. 4, groups 1 through 7, the transfer from CS to FCS of T24-transfected cultures was begun on day 4 after transfection and terminated on day 5, 6, 7, 8, 12, 16, or 24, respectively. In Fig. 4, groups 8 through 13, treatment with FCS was delayed until day 5, 6, 7, 8, 12, or 16, respectively. Maximum enhancement of cell transformation by FCS was seen in Fig. 4, groups 5, 6, and 7. It is of interest that group 1 through 7 showed approximately the same number of prominent,

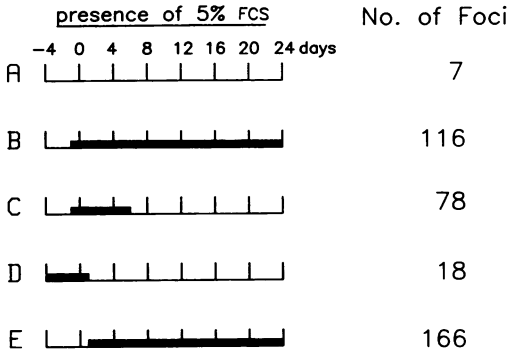

FIG. 3. Time course studies of the effect of 5% FCS on T24-induced transformation of C3H 10T1/2 cells. The heavy lines indicate the periods for which the cells were exposed to FCS. The numbers indicate the number of transformed foci obtained per 5 × 10^5 recipient cells.

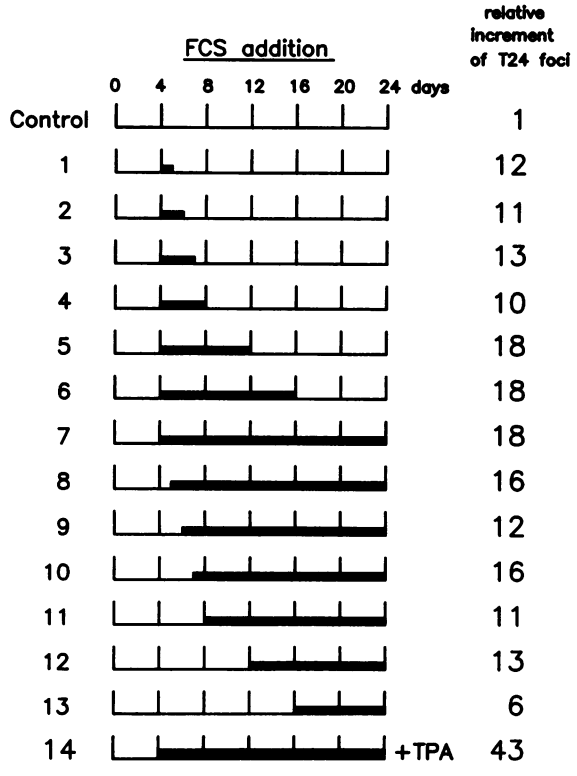

FIG. 4. Time course studies of the effect of FCS on T24-induced transformation of rat 6 cells. The heavy lines indicate the periods for which the cells were exposed to FCS. The data are expressed as the ratio of foci obtained in the presence of FCS to those obtained in the presence of CS (i.e., the control). The number of transformed foci in the control plate was six per 5 × 10^5 recipient cells.

TABLE 2. Effects of growth factors on T24-induced transformation of C3H 10T1/2 and rat 6 cells[a]

Addition	Final concn	Transformed foci (% of control) for:	
		C3H 10T1/2	Rat 6
None		100	100
TGF-β	0.01 ng/ml	32	ND[b]
	0.03 ng/ml	9	ND
	0.1 ng/ml	0	20
	0.3 ng/ml	0	40
TGF-α	1 ng/ml	55	ND
	2 ng/ml	23	ND
TGF-α (2 ng/ml) + TGF-β			
	0.01 ng/ml	17	ND
	0.03 ng/ml	0	ND
	0.1 ng/ml	0	ND
	0.3 ng/ml	0	ND
EGF	0.1 ng/ml	ND	20
	0.5 ng/ml	ND	70
	1.0 ng/ml	ND	10
	5.0 ng/ml	17	0
PDGF	0.1 U/ml	ND	120
Insulin	5 μg/ml	ND	100
	10 μg/ml	ND	90
Transferrin	10 μg/ml	ND	120
Selenium	5×10^{-7} M	ND	80
	5×10^{-6} M	ND	80
CR-ITS[b]		ND	170
Vitamin B_{12}	10^{-7} M	ND	100
	10^{-6} M	ND	170

[a] All assays were carried out in the presence of 5% CS, and the treatment was started 4 days after transfection and continued throughout the experiment.

[b] ND, Not done.

[c] ITS premix was obtained from Collaborative Research. Final concentrations: insulin, 25 μg/ml; transferrin, 25 μg/ml; selenium, 25 pg/ml.

small, dense foci at day 6. Thus the early withdrawal of FCS in groups 1 through 4 apparently causes regression of some T24-induced foci at day 6. Our results also indicate that the exposure of cells to FCS for just 1 day can exert an irreversible enhancement of focus formation (Fig. 4, group 1). In addition, we found that a sixfold enhancement of focus formation could be obtained even when treatment with FCS was postponed until day 16 after T24 transfection (Fig. 4, group 13). These results provide evidence that FCS exerts its effects at a stage after the integration of the transfected T24 DNA and that there are some cells in the transfected population which carry the activated oncogene but do not express the transformed phenotype unless they are exposed to FCS. An additional finding was that when TPA (100 ng/ml) was added to growth medium containing 5% FCS, it produced an additional two- to threefold increase in T24-induced foci over the effects of FCS alone (Fig. 4, compare group 14 with group 7). Thus FCS and TPA appear to act in an additive or synergistic manner with respect to enhancement of cell transformation.

Effects of specific growth factors on the formation of T24-induced foci. Serum contains a number of growth factors (10). To determine whether the focus-enhancing activity of FCS was due to the presence of known growth factors, various growth factors and compounds were tested in our standard transfection assay. The T24-transfected C3H 10T1/2 or rat 6 cells were grown in the presence of 5% CS. Each of the substances to be tested for activity was added to the cultures on day 4 after transfection and also during each refeeding with fresh growth medium, throughout the remainder of the experiment. None of the substances tested exerted significant enhancement of T24-induced focus formation (Table 2). We noted that at doses higher than 0.3 ng/ml, TGF-β was toxic to both C3H 10T1/2 and rat 6 cells. On the other hand, mouse EGF, but not TGF-α at the doses tested, stimulated cell growth. The EGF-treated rat 6 cultures became quite dense and the cells were refractile, and yet EGF did not enhance the yield of T24-induced foci. Therefore the enhancement obtained with FCS does not appear to be due to simple stimulation of cell growth.

Fractionation studies of FCS. In view of the above results, it was of interest to obtain information about the factor(s) present in FCS that stimulates T24-induced focus formation. To determine the size range of the factor(s), we subjected FCS to dialysis with 14,000- to 12,000-molecular-weight cutoff dialysis tubing, as described in Materials and Methods. When both the nondialyzable fraction and the dialysate were assayed on T24-transfected rat 6 cells, in the presence of 5% CS, we found that the nondialyzable fraction exhibited no focus-enhancing activity, whereas the dialysate contained the same focus-enhancing activity as an equivalent amount of the original FCS (Table 3). It is of interest that when the T24-transfected cultures were fed with dialyzed 5% CS (nondialyzable fraction) in the absence of CS, we obtained only 0.1 times as many foci as when the cultures were fed 5% undialyzed CS. This finding suggests that the active factor may also be present in small quantities in CS. The fact that a fraction obtained from FCS enhances focus formation when added to CS indicates that FCS and CS differ because FCS contains a positive acting factor, and not because CS contains an inhibitory factor. Further dialysis studies indicated that the active factor present in FCS passed through dialysis tubing with a 2,000-molecular-weight cutoff. We also found that the factor present in the dialysate was heat stable (Table 3), was stable at pH 2 or pH 12, and remained in the aqueous phase after extraction with either acetyl acetate or chloroform-methanol (2:1, vol/vol) (data not shown). Digestion of the dialysate with pronase (1,000 U/ml at 37°C for 2 h) did not destroy its activity, suggesting that the active factor

TABLE 3. Effect of serum components on T24-induced focus formation[a]

Serum components in medium	Relative increment of T24-induced foci[b]
CS	1.0
FCS	24.5
Dialysate of FCS + CS	22.0
Dialyzed CS	0.1
Dialyzed FCS	0.5
Dialysate heated at 100°C for 10 min	24.0

[a] CS and FCS were added to the growth medium at a 5% concentration on day 4 following transfection of rat 6 cells with T24 DNA. Fractions of CS or FCS were tested at amounts equivalent to 5% serum. For additional details see Materials and Methods.

[b] The data are expressed as the relative increase in foci when compared with those obtained with CS.

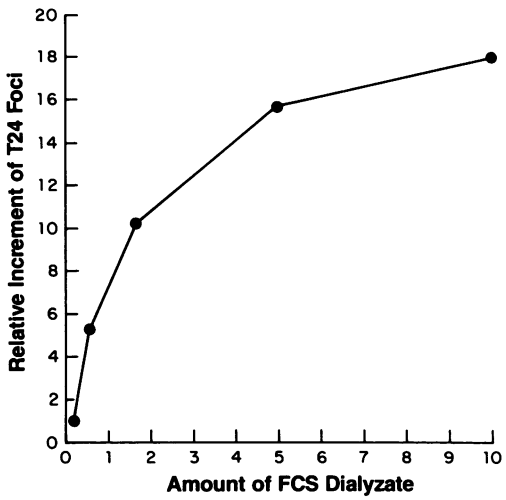

FIG. 5. Dosage dependence studies of the effect of the dialysate of FCS on T24-induced transformation of rat 6 cells. The doses of dialysate in the growth medium are expressed in terms of an equivalent percentage of unfractionated FCS.

may not be a polypeptide or that it is very resistant to proteolytic degradation.

The dialysate was then used to perform a dosage dependence study. The T24-transfected rat 6 cultures were grown in medium containing 5% CS and treated with increasing amounts of the FCS dialysate, beginning 4 days after transfection and continuing throughout the experiment. We found that the dialysate of FCS enhanced the formation of T24-induced foci in a dosage-dependent manner; the maximum effect was obtained with an amount of dialysate that was equivalent to 5% FCS (Fig. 5).

To further characterize this material, we subjected the dialysate fraction of FCS to gel filtration chromatography on a Bio-Gel P10 column with appropriate molecular weight markers. Assays of the fractions for enhancement of T24-induced foci revealed that the active material had a molecular weight of about 1,000 to 2,000 (Fig. 6). These findings are consistent with the above-described dialysis studies. In view of the low molecular weight of this material, we also assayed selenium, vitamin B_{12}, and triiodothyronine (Table 2; data not shown), but they did not produce the enhancing effects seen with the serum factor.

DISCUSSION

The present results indicate that FCS, and a low-molecular-weight factor isolated from FCS, markedly enhance the transformation of both C3H 10T1/2 and rat 6 fibroblasts transfected with the activated c-Ha-*ras* oncogene T24. These results extend our previous results, which were obtained with the potent tumor promoter TPA (11, 12). FCS is more effective than TPA, since in cultures fed with FCS, dense transformed foci could be seen as early as 6 days after T24 transfection, whereas in TPA-treated cultures, transformed foci were not seen until about 12 days. In addition, the T24-induced foci seen in FCS-treated cultures were larger, appeared more transformed, and had a distinctive morphology (Fig. 1).

We obtained three lines of evidence that the enhancement of T24-induced transformation is not due to increased cellular uptake or increased integration of the plasmid DNA. (i) In time course studies we found that even if FCS treatment was delayed until 12 days after the DNA transfection procedure, FCS produced a 6-fold enhancement in the number of transformed foci (Fig. 4). (ii) The Southern blot analyses of DNAs extracted from pT24 transformants isolated from C3H 10T1/2 and rat 6 cultures showed that there were no systematic differences in the number of integrated pT24 genes between clones obtained from the control and FCS-treated cultures (Fig. 2). (iii) FCS did not enhance the transfection efficiency of *gpt* or *neo* genes in our model systems (Table 1).

Since serum contains a variety of growth factors with mitogenic effects on various types of cells (10), it is conceivable that FCS enhances focus formation through nonspecific stimulation of the growth of C3H 10T1/2 and rat 6 cells. Under our culture conditions, however, FCS did not stimulate the growth of these cells. On the contrary, cells grown in FCS displayed a lower saturation density than those grown

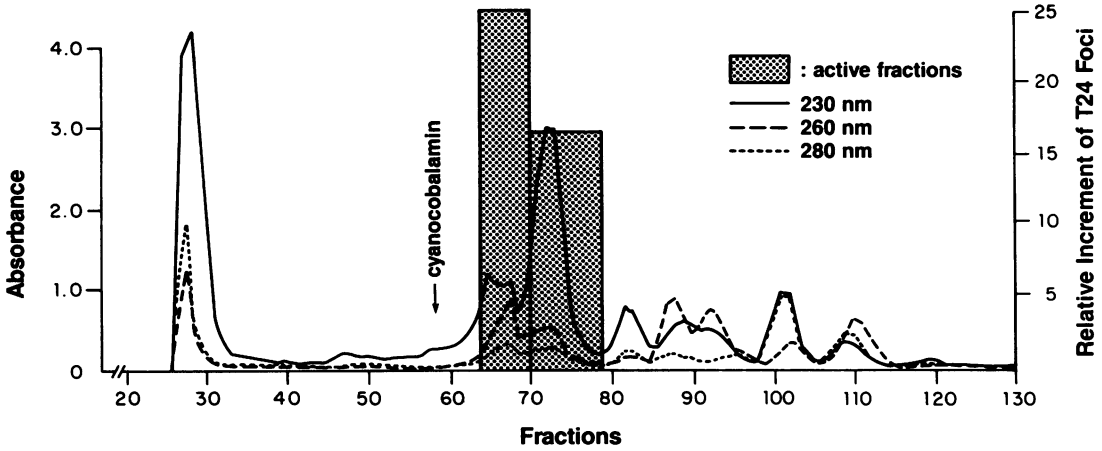

FIG. 6. Bio-Gel P10 chromatography of the dialysate of FCS. The dialysate of FCS was applied to a Biol-Gel P10 column (1.5 by 65 cm) and eluted with 0.01 M phosphate buffer (pH 7.0) at a rate of 5 to 8 ml/h. Fractions (2 ml) were collected, monitored for A_{230}, A_{260}, and A_{280}, and pooled as indicated. The pooled fractions were assayed for their ability to enhance focus formation in T24-transfected rat 6 cells, in the presence of 5% CS. The data obtained from the transfection assay are expressed as the ratio of foci obtained in the presence of each pool to those obtained in the presence of CS alone. For additional details, see Materials and Methods. The elution position of the molecular weight marker cyanocobalamin (molecular weight 1,300) is shown.

in CS (data not shown). Furthermore, the ability of FCS to enhance T24-induced focus formation was not seen with various mitogens, including EGF, PDGF, and insulin (Table 2).

Cotransfection studies have shown that the v-*myc* oncogene, when linked to the Moloney murine leukemia virus promoter, can synergize with an activated *ras* oncogene to cause transformation of early-passage rat embryo fibroblasts (13). These results suggest that FCS and TPA might act simply by enhancing the expression of the endogenous c-*myc* proto-oncogene in rat 6 cells. This does not appear to be the case, since treatment of rat 6 cells with either FCS or TPA, under the conditions used in our transformation studies, failed to produce a sustained increase in c-*myc* mRNA levels. Furthermore, we found that FCS was able to cause a further enhancement of focus formation in cells cotransfected with v-*myc* and T24 DNA, suggesting that it acts by a mechanism different from that of the *myc* gene (unpublished data).

Any interpretation of the effects we have observed with FCS is complicated by the fact that the precise mechanism of action of the p21 proteins encoded by the *ras* oncogenes is not known. Since these proteins bind GTP and bear homology to the α subunits of certain G proteins, and since they clearly alter growth control, it seems likely that their action is similar to that of some of the known G proteins in terms of modulating the activity of an enzyme involved in signal transduction (15). There is indirect evidence that they might act by modulating the turnover of phosphatidylinositol-4,5-bisphosphate and thus control the generation of the signal molecules diacyglycerol and inositol triphosphate (6). Investigators in our laboratory have obtained evidence that the p21 proteins can also modulate the phosphorylation of specific mitochondrion-associated proteins (2). Since TPA causes a direct activation of protein kinase C (3), the ability of TPA to enhance cellular transformation in T24-transfected cells might reflect a summation of effects at the level of phosphorylation of critical proteins that control cell growth. Further studies are required to determine whether the low-molecular-weight factor from FCS, which we have identified in the present study (Fig. 6), also acts through protein kinase C or other protein kinases. Studies are in progress to purify this factor to homogeneity and to identify its precise structure. This will facilitate studies on its mechanism of action.

The results obtained in the present study may have considerable biologic importance, since they indicate that FCS normally contains a factor that markedly enhances the transformation of cells carrying an activated oncogene. This finding may provide insights into endogenous factors that enhance the multistage carcinogenic process following the mutational activation of specific oncogenes.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA 02656 from the National Cancer Institute and by the Alma Toorock Memorial for Cancer Research.

We thank Michael Wigler, Richard Axel, and Mitchell Goldfarb for providing plasmids pT24, pIBW, pMuLV-myc, and c-*myc* cDNA. We also thank Michael Sporn and R. Lehrman for providing TGFα, TGFβ, and PDGF. We also express our appreciation to Nancy Mojica for valuable assistance in the preparation of this manuscript.

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